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Calculation of the Rate of Absorption of Exogenous Creatinine.

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The purpose of this paper is to calculate the rate of absorption of a substance from the changes in the concentration of the substance in the blood.

Because the rate of absorption is in a large measure independent of the chemical changes which the substance may undergo after absorption, we shall study the absorption of a substance which is not metabolized in the body—creatinine. (To avoid repetition, the word creatinine will be used to mean exogenous creatinine, unless otherwise stipulated). The conclusion that creatinine is not metabolized is based on previous work showing that (1) after intravenous injection, about 96% of creatinine is recovered in the urine in the first 24 hours and (2) after oral administration to a patient with an ileal fistula, the amount of creatinine recovered in the urine was about equal to the difference between the quantity given and the amount collected from the fistula.[1] If this conclusion is valid, it follows that the incomplete recovery of creatinine in oral experiments (a fact ascertained by all investigators) is due to incomplete absorption from the intestinal tract. In other words, the amount of creatinine recovered after ingestion is actually the amount absorbed.

After ingestion, the concentration of creatinine in the plasma rises to a maximum and then falls. The rate of this change in concentration depends, in general, on three other rates: the rate of absorption from the intestine, the rate of excretion by the kidney, and the rate of diffusion from the plasma into the other body fluids. If the body fluids are in diffusion equilibrium during absorption, the rate of diffusion may be eliminated from further consideration. Because of this simplification we shall limit this paper to experiments in which the assumption of diffusion equilibrium can be justified. The method of calculation when the rate of diffusion must be considered will be presented in another paper.

In the first few minutes of absorption the concentration of creatinine in some parts of the portal system is undoubtedly higher than

1 Dominguez, R., and Pomerene, E., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 26.

in others and especially higher than in the systemic veins. This initial difference, however, becomes negligible after a short time because of the circulation of the blood and because of the dilution of the blood from the portal system with the larger volume of blood from the venæ cavæ. Inasmuch as the absorption of creatinine usually lasts several hours, we shall neglect the transient initial inequalities in concentration.

With the foregoing considerations in view, the absorption of creatinine may be formulated as follows: Let q be the amount of creatinine which is present in the body at a given time, an amount which, by assumption, is evenly distributed in the body fluids. The rate of change in the amount of creatinine q is equal to the difference between the rate of absorption, ρ, and the rate of excretion, η, that is,

$$\frac{dq}{dt} = \rho - \eta \qquad (1)$$

The amount q is equal to the product of creatinine concentration in the plasma, ξ, and the volume of distribution, V. Since in the conditions of the experiment the volume V remains constant, equation 1 becomes

$$V\frac{d\xi}{dt} = \rho - \eta \qquad (2)$$

or, after transposition,

$$\rho = V\frac{d\xi}{dt} + \eta \qquad (3)$$

Equation 3 means that, at any given time, the rate of absorption is equal to the product of the volume of distribution and the rate of change of the plasma concentration, plus the rate of excretion.

The rate of excretion, η, is, on an average, proportional to the plasma concentration, ξ, that is,

$$\eta = A\xi \qquad (4)$$

where A is a constant.[2]

With this value of η, equation 3 becomes

$$\rho = V\frac{d\xi}{dt} + A\xi \qquad (5)$$

After absorption ends (that is, $\rho = 0$ in equation 5), the creatinine concentration in the plasma falls according to the equation[3]

$$\xi = a\,e^{-\alpha t} \qquad (6)$$

where a is a constant, e is the base of natural logarithms and α represents the relation

$$\alpha = \frac{A}{V} \qquad (7)$$

Equation 6 has been experimentally verified.[2]

The calculation of the rate of absorption ρ (equation 5), consists of the following steps. (1) The rate of excretion is plotted *vs.* the plasma concentration. The slope of the line is the constant A. (2) The logarithms of the plasma creatinine concentrations are plotted *vs.* time and the constants of equation 6 are computed. The slope of the linear descending part of the graph is the constant α. The plasma data can be increased in number by dividing the rates of excretion of creatinine by the constant A and treating these data as plasma data. For this step, the endogenous blanks are subtracted from both the plasma concentration of total creatinine and the rate of excretion of total creatinine. (3) A smooth curve is drawn through or fitted to the early plasma creatinine data and is joined smoothly to the exponential curve $a\,e^{-\alpha t}$ of step 2. This curve should begin at zero with the slope zero because, at zero time, both the rate of absorption and the rate of excretion are zero. Hence, by equation 3, the slope $d\xi/dt$ of the plasma curve is zero at zero time. (4) A sufficient number of values of ξ and $d\xi/dt$ are calculated from the fitted curve. (5) the volume V is calculated by equation 7. (6) With these values, the operations indicated in the right hand side of equation 5 are performed. The curve of the rate of absorption is then plotted *vs.* time.[4]

[2] Dominguez, R., and Pomerene, E., *J. Biol. Chem.*, 1934, **104**, 449.

[3] Dominguez, R., PROC. SOC. EXP. BIOL. AND MED., 1934, **31**, 1146.

[4] For further details the following books may be consulted: Lipka, J., *Graphical and Mechanical*

TABLE I.

Protocol of Experiments on Subject G.N.B.

Breakfast at 7:45 a.m.; creatinine, 8 g in water, taken by mouth at 9:17 a.m.; lunch at 11:50 a.m.; dinner at 5:00 p.m.

Urine		Blood	
Interval of collection	Vol., cc	Creatinine conc. mg/100 cc	Time	Creatinine conc., mg/100 cc of oxalated plasma
9:09 a.m.-10:27 a.m.	79	671	10:22 a.m.	7.8
10:27 " -11:29 "	70	1669	11:24 "	11.9
11:29 " -12:35 p.m.	71	1744	12:29 p.m.	9.1
12:35 p.m.- 1.37 "	43	1745	1:31 "	6.3
1:37 " - 2:40 "	73	869	2:34 "	4.8
2:40 " - 4:14 "	308	213	4:04 "	3.3
4:14 " - 5:55 "	76.5	807	5:51 "	3.0
5:55 " - 7:09 "	60	561	7:04 "	2.8
7:09 " - 9:20 a.m. the following morning	1113	184		
9:20 a.m.-10:26 a.m.	22.5	316		

Ileal fistula		
Interval of collection	Vol. cc	Conc. of chromogenic substances mg creatinine per 100 cc
9:10 a.m.-10:29 a.m.	318	4.2
10:29 " -11:30 "	185	34.2
11:30 " -12:36 p.m.	132.5	1013
12:36 " - 1:38 "	137	160
1:38 " - 2:41 "	310	94
2:41 " - 4:10 "	300	12.5
4:10 " - 5:56 "	298	11.3
5:56 " - 7:10 "	220	6.8

The amount absorbed can be obtained by calculating the area under the graph of the rate of absorption. The amount excreted can be determined, first, by calculating the area under the plasma creatinine curve and then by multiplying the area thus found by the constant A. If the excretion data are regular and the constant A applies to the whole curve, the calculated amount excreted will be close to the amount actually excreted. If the excretion data are irregular and the constant A is not well determined, an adequate value of A can be obtained by dividing the amount actually found by the area under the plasma creatinine curve. This step follows from the integration of equation 4.

Computation, John Wiley and Sons, Inc., New York, 1918; Scarborough, J. B., *Numerical Mathematical Analysis*, The Johns Hopkins Press, Baltimore, 1930; Sherwood, J. K., and Reed, C. E., *Applied Mathematics in Chemical Engineering*, McGraw-Hill Book Company, Inc., New York, 1939.

Since the calculated amount absorbed is equal to the calculated amount excreted, the equality of the calculated amounts serves as a check of the numerical work.

With respect to diffusion equilibrium, it follows from the meaning of the exponent a (equation 7) that if A and a are constant, the volume of distribution V is also constant. This constant value of V holds for the descending part of the plasma creatinine curve, the part from which a is calculated (equation 6). A subsidiary calculation is necessary to test whether the same value of V holds also for the first part of the plasma creatinine curve. If it does, the assumption of diffusion equilibrium holds.

All these steps will be illustrated by several examples.

Subject G.N.B. White male, age 24, weight 54 kg, height 179 cm. This man had a permanent ileostomy for a chronic ulcerative colitis.[1] The protocol of the experiment is

presented in Table I. All the analyses were made according to the methods of Folin[5] and Folin and Wu.[6] The average endogenous creatinine in the plasma was 0.74 mg per 100 cc and the average rate of excretion of endogenous creatinine was 1.0 mg per min. The relation of the rate of excretion of total creatinine to the total plasma creatinine after the maximum concentration is shown in Fig. 1. By

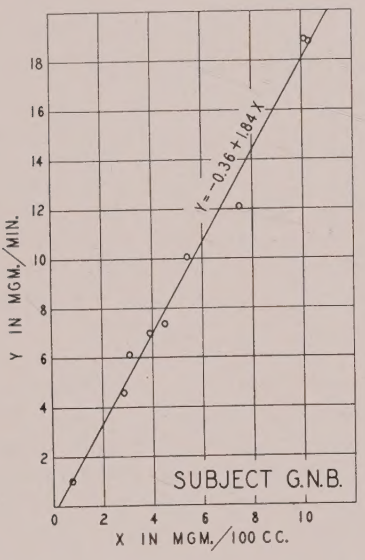

FIG. 1.

Relation between the rate of excretion of total creatinine y and the plasma concentration of total creatinine x from the data of Subject G.N.B. after the maximum creatinine in the plasma (Table I).

the method of averages, the slope of the line, constant A, is 1.841. After subtraction of the endogenous blanks, the rates of excretion were multiplied by 1/1.841 and all the data were treated as plasma creatinine. The plot is shown in Fig. 2. A smooth curve was drawn through the points from 0 to 4.2 hr. The line $ln\ \xi = ln\ 17.89 - 0.259\ t$ was fitted to the points from 4.2 to 9.25 hr by the method of averages. The whole curve can be seen in Fig. 2. From a magnified plot of this curve, values and differences of the concentration ξ were tabulated from 0 to 2.5 hr at intervals of 0.1 hr and from this table the values of the

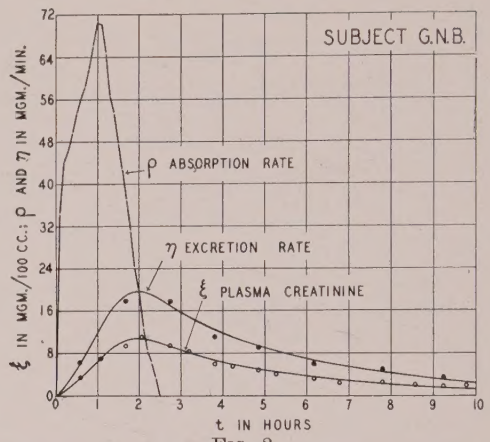

FIG. 2.

Curves of the plasma creatinine ξ, of the rate of excretion η, and of the rate of absorption ρ from the data of Subject G.N.B. (Table I). The closed circles represent the average rate of creatinine excretion at the middle of the intervals of collection and the open circles represent the concentrations of plasma creatinine.

derivative $d\xi/dt$ were computed. The volume V, corrected for units, is $V = \frac{1.841}{0.259} \times 60 \times 10^2$ cc $= 42.6 \times 10^3$ cc. The rate of absorption was then calculated by equation 5 and plotted (Fig. 2). The irregularities in the graph are undoubtedly due to uncertainties in the tabulated values of ξ.

The test for diffusion equilibrium is as follows. The term-by-term integration of equation 5, from 0 to t_1, is

$$P_1 = V\xi_1 + H_1$$
$$(\rho_1 = V\xi_1 + \eta_1)$$

Where P_1 is the amount absorbed in the time t_1 and H_1 is the amount excreted in the same time. This equation simply means that the amount absorbed in a given interval (P_1) is equal to the amount present in the body fluids at the end of this interval ($V\xi_1$) plus the amount excreted in the same interval (H_1). The calculation is carried out at cumulative intervals as shown in Table II. It is seen in this table that the cumulative amounts absorbed have the mean value 5.92 g from 2.2 to 9.87 hr and that the variations in the calculated amount absorbed lie within 2.2% of the mean. The only constant quantity in this calculation is the volume V, a volume which was

[5] Folin, O., *J. Biol. Chem.*, 1914, **17**, 469.

[6] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, **38**, 81.

TABLE II.
Cumulative Amount of Creatinine Absorbed by Subject G.N.B.

(1) Time t hr	(2) Plasma creatinine (from graph) ξ mg/100 cc	(3) Volume of distribution V liters	(4) Amt of creatinine present in body fluids at time t $V\xi$ g	(5) Amt excreted in time t (observed) g	(6) Amt absorbed in time t (4) + (5) g
1.17	7.8	42.6	3.32	0.44	3.76
2.20	10.5		4.47	1.55	6.02
3.30	7.8		3.32	2.72	6.04
4.33	5.8		2.47	3.41	5.88
5.38	4.4		1.87	3.98	5.85
6.95	3.0		1.28	4.54	5.82
8.63	1.9		.81	5.06	5.87
9.87	1.4		.60	5.32	5.92

The mean of the last 7 figures of column 6 is 5.91 g.

obtained from the part of the plasma data in which equilibrium can be assumed. Since the use of this value of V in the first part of the experiment does not lead to any internal inconsistency, the assumption of diffusion equilibrium is justified.

The amount absorbed, calculated by Simpson's rule, is 6.13 g. The amount excreted, calculated by numerical integration from 0 to 4.2 hr and by straight integration from 4.2 to 25 hr, is 5.93 g. The amount actually excreted from 0 to 25 hours, from the data of Table I, is 6.53 g. Inasmuch as the calculation of the rate of absorption was based on the rates of excretion of the first 10 hours only, some adjustment is needed to take care of the amount excreted after 10 hours. But since this adjustment does not change the shape of the early part of the plasma creatinine curve, we have preferred to present the results without adjustment. We regret that, for reasons already given,[1] this experiment could not be repeated.

It should be noticed that (1) the maximum rate of absorption (Fig. 2) corresponds to the first point of inflexion of the plasma creatinine curve and (2) at the maximum plasma creatinine, the rate of absorption is equal to the rate of excretion. At the time of the maximum plasma creatinine, however, 5.89 g has already been absorbed.

In this subject we have 3 estimates of the amount absorbed: (1) by the difference between the quantity ingested and the amount collected from the fistula, 8 g — 1.96 g = 6.04

TABLE III.
Protocol of Experiment on Subject S.
Breakfast at 7:45 a.m.; creatinine, 8 g in water, taken by mouth at 9:07 a.m.; lunch at 12:30 p.m.; dinner at 5:30 p.m.

Urine			Blood	
Interval of collection	Vol., cc	Creatinine conc., mg/100 cc	Time	Creatinine conc., mg/100 cc oxalated plasma
8:05 a.m.- 9:04 a.m.	23.0	260	9:06 a.m.	.8
9:04 " - 9:39 "	61.5	368	9:41½ "	8.2
9:39 " -10:10 "	103.5	520	10:13 "	11.0
10:10 " -11:08 "	78.0	1337	11:11½ "	11.2
11:08 " -12:09 p.m.	152.0	656	12:13 p.m.	9.1
12:09 p.m.- 1.07 "	73.0	912	1:12 "	6.4
1:07 " - 2:11 "	52.5	945	2:15 "	5.7
2:11 " - 3:07 "	44.5	863	3:11 "	4.4
3:07 " - 4:09 "	53.5	652	4:11 "	3.3
4:09 " - 5:08 "	61.0	521	5:13 "	3.0
5:08 " - 9:19 a.m. the following morning	780.0	239	9:24 a.m.	.88
9:19 a.m.-10:20 a.m.	55.0	116		

g, (2) by the amount excreted in 25 hr and the assumption that ingested creatinine is not transformed in the body, 6.53 g, and (3) by the method of this paper, 6.13 g. The first estimate is independent of any observations on plasma or urine. The second estimate requires no knowledge of the rate of excretion and is of course independent of the plasma creatinine concentration. The third estimate depends only on the rates (equation 5) and is independent of the amount excreted after, say, 10 hr. These 3 estimates represent 76, 82, and 77% of the quantity ingested. With this approximation we can say that the amount excreted is equal to the amount absorbed, or, in other words, that ingested creatinine is not transformed in the body.

Subject S. White male, age 29, weight 56.2 kg, height 163.2 cm. The protocol of the experiment is given in Table III. The endogenous creatinine excretion of several control periods was, on an average, 1.0 mg per minute. The endogenous plasma creatinine was 0.8 mg/100 cc. The relation between the rate of excretion and the plasma concentration of total creatinine is seen in Fig. 3.

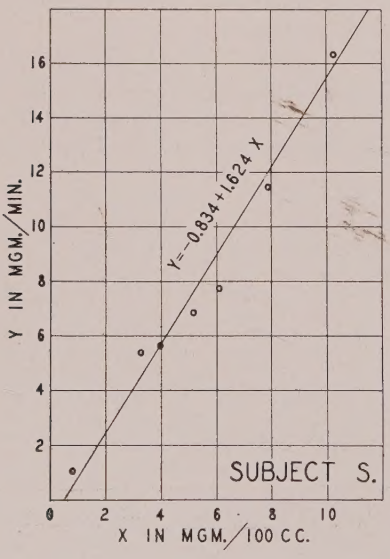

FIG. 3.

Relation between the rate of excretion of total creatinine y and the plasma concentration of total creatinine x from the data of Subject S. after the maximum creatinine in the plasma (Table III). The line with slope 1.624 (see text) passes through the centroid of the points shown in the graph.

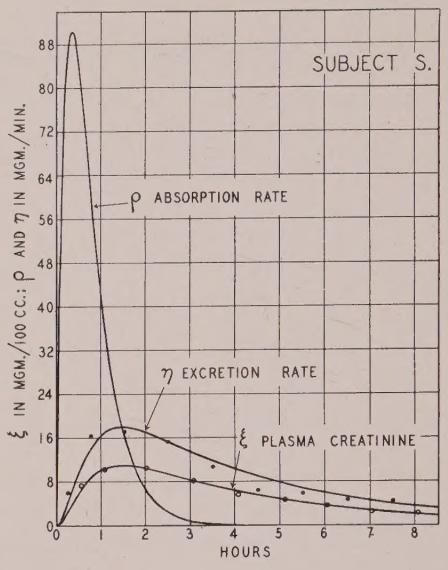

FIG. 4.

Curves of the plasma creatinine ξ (equation 8), of the rate of excretion η (equation 4), and of the rate of absorption ρ (equation 9) from the data of Subject S. (Table III). The closed circles represent the average rate of creatinine excretion at the middle of the intervals of collection and the open circles represent the concentrations of plasma creatinine. The values of the constants of the equations are shown in Table IV.

Instead of drawing a smooth curve through the points from 0 to 3 hr, as in the first example, a mathematical equation was fitted to all the points. The equation is

$$\xi = a e^{-\alpha t} - c e^{-\gamma t} + d e^{-\delta t} \qquad (8)$$

where the first exponential fits the data from 3 to 8 hr, the second fits the residuals of the first exponential and the data from 1 to 3 hr, and the third exponential forces the curve to start at zero with the slope zero.

The equations for the latter conditions are

$$a - c + d = 0$$
$$a\alpha - c\gamma + d\delta = 0$$

The constants d and δ are computed from these equations as soon as the other 4 constants are determined.

The values of the constants are given in Table IV and the curve of the plasma creatinine is reproduced in Fig. 4.

The rate of absorption ρ is derived by per-

TABLE IV.
Constants of Equations 4, 8, and 9, and Quantities of Creatinine Ingested, Excreted, and Absorbed.

Subject and exp. No.	Constants of Equation 4	Constants of Equation 8					Constants of Equation 9	Vol. of distribution, V	Quantity ingested	Amt excreted in stated time		Calculated amount absorbed,
	A	a	c	d	α	γ	δ	p				
	cc/min $\times 10^2$	mg/cc $\times 10^{-2}$				l/hr		mg/min	liters	g	hr	g	g
GNB*	1.84	20.8			0.296				42.6	8	25.2	6.52	6.13
S	1.62	19.4	38.6	19.2	0.278	2.04	3.8	396	35.0	8	25.2	5.43	5.42
E3†	1.81	6.0	6.7	0.7	0.286	1.09	8.0	33.8	38.0	3	8.3	1.41	1.64
E4	1.99	18.3	25.5	7.2	0.286	1.69	5.3	248	41.7	8	6.7	4.87	5.99
E5	1.98	10.8	15.0	4.2	0.286	1.69	5.3	145	41.5	5	10.2	3.27	3.53
E6	1.94	11.0	15.4	4.4	0.286	1.69	5.2	146	40.7	5	8.6	3.13	3.52
E8	1.67	12.0	17.4	5.4	0.286	1.64	4.6	137	35.0	5	7.2	2.73	3.26

* This subject had a permanent ileal fistula.[1] During this experiment 1.96 g of creatinine was recovered from the fistula (Table I).
† Data on Subject E from Dominguez, R., and Pomerene, E., *J. Biol. Chem.*, 1934, **104**, 449.

forming on equation 8 the operations indicated in equation 5. The result is

$$\rho = p(e^{-\gamma t} - e^{-\delta t}) \qquad (9)$$

where $p = \frac{Ac}{a}(\gamma - a) = \frac{Ad}{a}(\delta - a)$.

The value of A for the descending part of the curves is, by the method of averages, 1.49. The value of A obtained by dividing the amount actually excreted in 25.2 hr by the area under the plasma creatinine curve is $A = 5.43/3.352 = 1.62$ (Fig. 3). This result indicates that the value of A was greater than the mean before the maximum plasma creatinine and smaller than the mean after the maximum. With $A = 1.62$, the curve of the rate of excretion (equation 4) and the curve of the rate of absorption (equation 9) are reproduced in Fig. 4. The amount absorbed is

$$\int_0^\infty \rho\, dt = 60 \times 396\left(\frac{1}{2.04} - \frac{1}{3.82}\right) = 5.42 \text{ g},$$

a good check of the numerical work (Table IV).

The maximum rate of absorption can be shown to occur at the time of the first point of inflexion of the plasma creatinine curve (Fig. 4). The rate of absorption is equal to the rate of excretion at the time of the maximum plasma creatinine, but by this time 4.905 g has already been absorbed.

The test for diffusion equilibrium is presented in Table V. The calculated amount absorbed reaches the value 5.52 g, its greatest value, 3.02 hr after ingestion (Table V) and this value is within 2% of the total amount excreted in 25.2 hr (Table IV). From 3 to 8 hr the amount absorbed remains stationary (Table V), except for small variations due to irregularities in excretion. As in the preceding example, the assumption of diffusion equilibrium is justified.

Subject E. White female, age 26, weight 45 kg, height 161 cm. The data of 6 experiments on this subject have been published elsewhere,[2] but in that publication only the data of the descending part of the curves were utilized. We have found that equation 8, which fits

TABLE V.
Cumulative Amount of Creatinine Absorbed by Subject S.

(1) Time t hr	(2) Plasma creatinine (from graph) ξ mg/100 cc	(3) Vol. of distribution V liters	(4) Amt of creatinine present in body fluids at time t $V\xi$ g	(5) Amt excreted in time t (observed) g	(6) Amt absorbed in time t (4) + (5) g
.525	6.0	35.0	2.10	0.19	2.29
1.04	10.3		3.60	0.70	4.30
2.01	10.5		3.68	1.68	5.36
3.02	8.3		2.90	2.62	5.52
3.99	6.3		2.20	3.22	5.43
5.06	4.7		1.64	3.66	5.30
5.99	3.6		1.26	3.98	5.24
7.02	2.7		.94	4.27	5.22
8.01	2.1		.74	4.53	5.26

The mean value of the last 6 figures of column 6 is 5.33 g.

the data of subject S, fits also the data of subject E.

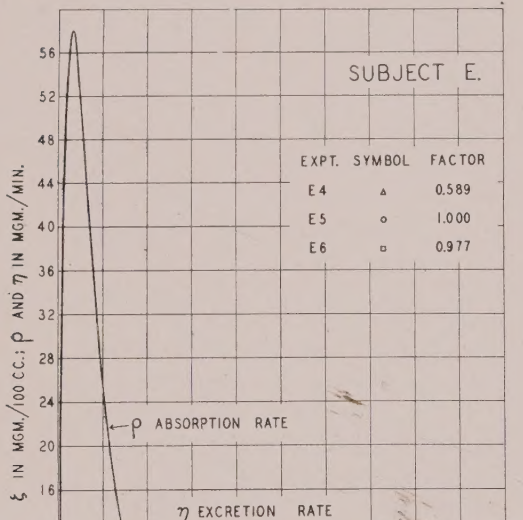

FIG. 5.

Curves of the plasma creatinine ξ (equation 8), of the rate of excretion η (equation 4), and of the rate of absorption ρ (equation 9) of Subject E. The data of three experiments have been reduced to one by means of the factors shown in this table. The values of the constants of the equations are shown in Table IV. The closed symbols represent the average rates of creatinine excretion at the middle of the intervals of collection and the open symbols represent the concentrations of plasma creatinine. (Data of Dominguez, R., and Pomerene, E., *J. Biol. Chem.*, 1934, **104**, 449.)

Of the 6 experiments on subject E, 5 form a homogeneous set with the common value $a = 0.286$. Furthermore, experiments E4, E5, and E6 can be reduced to one—as shown in Fig. 5—by means of the factors 0.589, 1, and 0.977, respectively. The other two experiments, E8 and E3, must be computed separately. The values of the constants in these five experiments, calculated by the present method, are given in Table IV. In order to make use of all the data and at the same time satisfy the amounts excreted before, as well as after the maximum plasma creatinine, a new value of A was calculated by dividing the amount actually excreted by the area under the plasma creatinine curve (Table IV).

The curves corresponding to the grouped data of experiments E4, E5, and E6 are reproduced in Fig. 5.

Discussion. The method we have described is applicable to substances which are in diffusion equilibrium during absorption, are not utilized in the body and are almost entirely excreted by the kidneys. It is also applicable to substances which are partially utilized and whose rate of utilization is proportional to the concentration of the substance in the plasma, such as xylose.[7,8]

With suitable modifications the method can be extended to more complicated problems in absorption.

[7] Dominguez, R., and Pomerene, E., *J. Clin. Invest.*, 1934, **13**, 753.

[8] Dominguez, R., Goldblatt, H., and Pomerene, E., *Am. J. Physiol.*, 1937, **119**, 429.

Unlike other methods used in physiology,[9] the present method determines the instantaneous rate of absorption. It can be used repeatedly in the same animal and is probably the only method that can be used in man. It throws a different light on the interpretation of the cumulative average absorption as calculated by Cori's method.[10] Thus, in the experiment on Subject G.N.B., the amount absorbed in 1 hr is 3.06 g, in 2 hr, 5.89 g, and in 3 hr, 6.13 g. Cori's coefficients are $3.06/1 = 3.06$, $5.89/2 = 2.95$, and $6.13/3 = 2.04$ g per hr. According to the usual interpretation, the rate of absorption of creatinine would have been considered constant in the first 2 hours. A glance at Fig. 2 shows that this conclusion would have been erroneous. In other words, the cumulative average amount absorbed per hour (Cori's coefficient) should not be considered the same as the rate of absorption.

Summary and Conclusions. For an inert substance in diffusion equilibrium, the rate of absorption at any time is shown to be equal to the rate of excretion plus the product of the volume of distribution and the rate of change in the concentration of the substance in the plasma. Two solutions of this equation are described and are applied to the absorption of creatinine in 3 human beings.

9 McGee, H. E., *Physiol. Rev.*, 1930, **10**, 473.
10 Cori, C. F., *J. Biol. Chem.*, 1925, **66**, 691.

15131

Effect of Pyridoxine on Granulopenia Caused by Thiouracil.*

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In the course of an investigation of the effect of thiouracil on the basal metabolic rate of 96 hyperthyroid subjects[1] we encountered a marked fall in the granulocytic cells of 20% of these patients among which were 4 cases of true agranulocytosis. It has been noted by Bilter, Schiro and Spies[2] that intravenous injection of pyridoxine into 3 pellagrins with pernicious anemia resulted in an increase in the white cell count and that this increase took place principally in the granulocytic series. Cantor and Scott[3] have administered pyridoxine hydrochloride intravenously in daily doses of 125-200 mg to 3 cases of agranulocytosis of varied origin and have noted a marked and rapid return to a normal granulocyte count in each instance.

Although thiouracil reduced the B.M.R. in our hyperthyroid subjects at an average rate of 1 unit % per day, with parallel amelioration of all accompanying signs and symptoms of thyrotoxicosis, it had to be withdrawn forthwith after a rapid or precipitate fall in the white count. We therefore used pyridoxine in the hope of lowering the incidence of these hematological accidents.

To 8 hyperthyroid subjects receiving the usual dose of thiouracil 200 mg of pyridoxine were given daily by mouth. Fig. 1 shows the total leucocyte count for 8-week periods prior (open symbols), and during (solid symbols) pyridoxine administration. The average white counts were increased between 1500-2000 cells per cu mm.

Fig. 2 shows the leucocyte count of a subject receiving a 0.2 g maintenance dosage of thiouracil, in whom owing to the destructive action of thiouracil on the bone marrow, the granulocyte count dropped from 3700 to 400 cells per cu mm within 2 days. Four cc of a 5% solution of pyridoxine hydrochloride (200 mg) were injected and resulted in an increase

* Thiouracil and pyridoxine supplied by Dr. Staunton Hardy of Lederle Lab.

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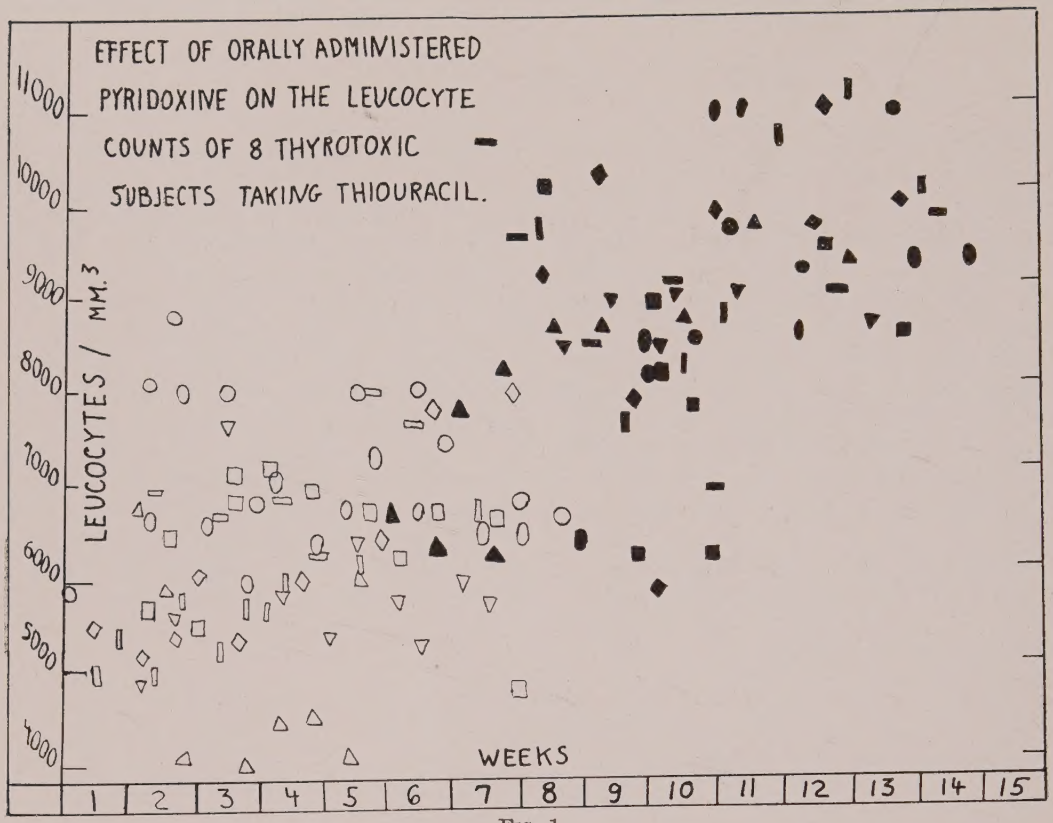
EFFECT OF ORALLY ADMINISTERED
PYRIDOXINE ON THE LEUCOCYTE
COUNTS OF 8 THYROTOXIC
SUBJECTS TAKING THIOURACIL.
LEUCOCYTES / MM.3
11000
10000
9000
8000
7000
6000
5000
4000
WEEKS
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15

FIG. 1.

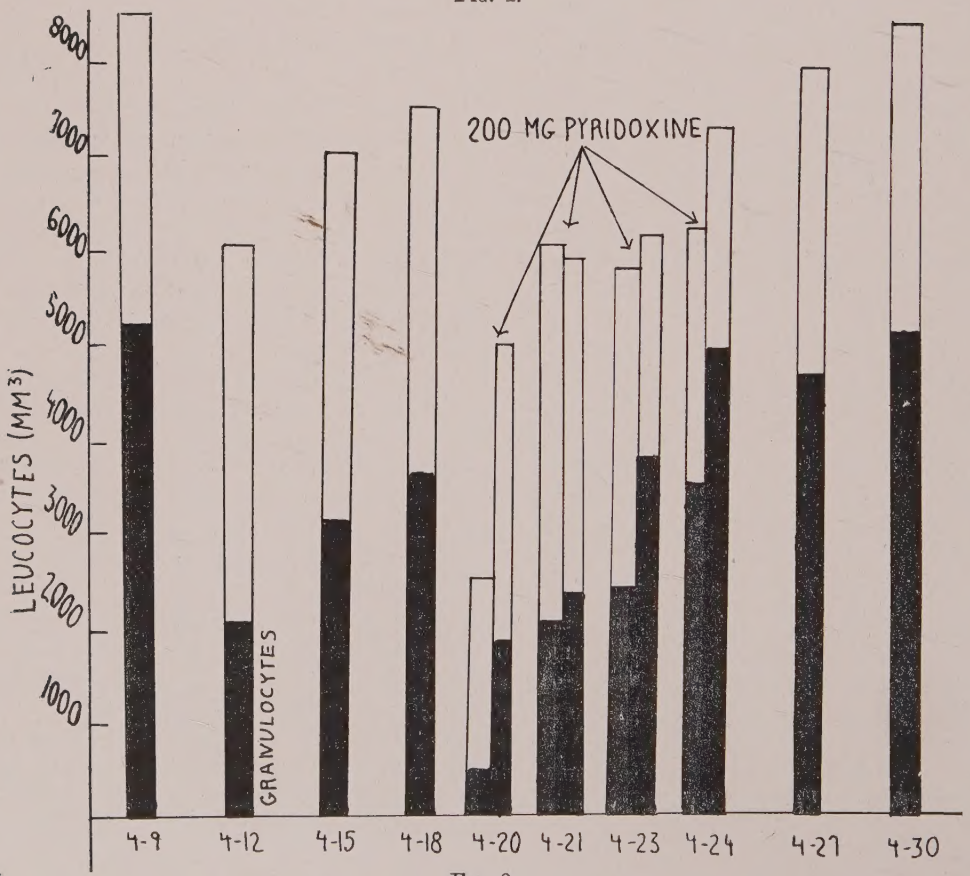
200 MG PYRIDOXINE
LEUCOCYTES (MM3)
8000
7000
6000
5000
4000
3000
2000
1000
GRANULOCYTES
4-9
4-12
4-15
4-18
4-20
4-21
4-23
4-24
4-27
4-30

FIG. 2.

CHART I.
Differential Leucocyte Count in a Thiouracil-sensitive Subject.

Date.	Thiouracil g	Pyridoxine mg intrav.	Leucocytes per mm³	Polys per mm³	% Polys	% Lymphocytes	% Monocytes	% Eosin.	% Staff
3/28	.6	0	13.500						
4/ 2	.6	0	13,500						
5	.6	0	11,200						
9	.6	0	6,550	3,210	49	46	3	2	
12	.3	0							
19	.3	0	7,800						
23	.3	0	6,600	2,508	38	54	6	1	2
26	.2	0	2,000	500	25	63	8	3	1
26	0	200	4,000	1,520	28	58	7	2	
27	0	200	5,750	1,610	28	60	6	4	
27	0	200	5,150	1,751	34	55	6	4	
28	0	200	6,000	2,400	40	57	5	2	2
29	0	100	7,500	3,300	44	47	1	5	1
5/ 4	0	0	7,900	4,101	52	43	1	1	2
5	0	0	12,250						
28	0	0	9,500						
31	.2	0	9,650						
6/ 4	.2	0	7,600						
7	.2	0	3,600	648	18	73	5	2	2
7	0	200	5,900	2,301	39				
8	0	200	7,000	3,010	43	50	3	1	3
9	0	200	8,950	4,674	52	43	3	1	1

to 1800 granulocytes per cu mm within 2 hours. This was repeated on 4 successive days (thiouracil being withdrawn) with an increase of the granulocyte count to 4900 cells per cu mm.

In Fig. 3 the curves of the granulocyte counts in 2 cases of agranulocytosis due to thiouracil are drawn on the same coordinates. In curve II no pyridoxine was used and there ensued the usual picture of agranulocytic angina with high fever and a necrosing lesion of the tonsillar area. In curve I 4 cc of a 5% solution of pyridoxine were injected at the points shown by arrows. Within 48 hours the granulocytes had increased to the same level reached in 13 days in the subject treated with transfusions and penicillin.

Chart I shows the blood picture in a 28-year-old man, obviously sensitive to thiouracil, in whom the white blood count had been 10,000 cells for the previous 2 weeks while taking 0.6 g of thiouracil daily. The count fell suddenly to 6,500 leucocytes per cu mm and the thiouracil was reduced to 0.3 g daily. The leucocyte count rose a little but on April 26 suddenly fell to 2000 with only 25% polymorphonuclear cells. After withdrawing thiouracil an injection of 200 mg of pyridoxine doubled the leucocyte count and tripled the granulocyte count within 4 hours. On May 31 an attempt was made to resume thio-

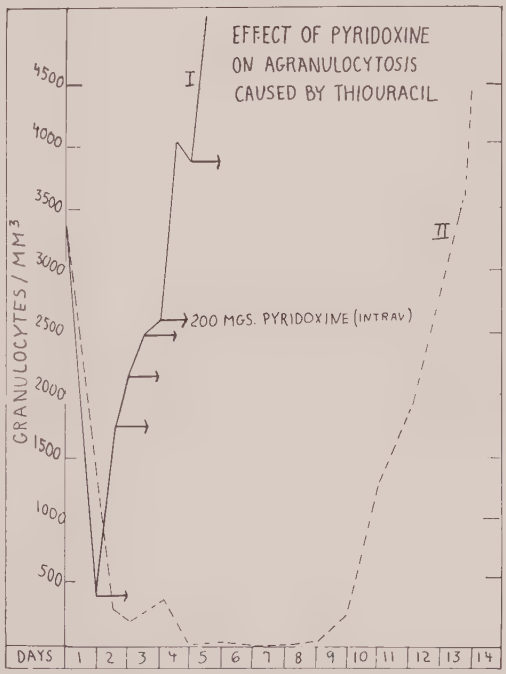

FIG. 3.
Solid line with pyridoxine. Broken line without pyridoxine.

uracil therapy with an initial small dose of 0.2 g daily but the leucocyte count fell within a week to 3,600 with 18% polys.

The appearance of the normal number of granulocytes in the blood stream is dependent on two factors: a hypothetical and as yet undemonstrated substance which regulates the orderly maturation of the myeloblast into the completely segmented granulocyte and the second which governs the entrance of the mature cell into the peripheral blood stream. The rapidity of action of the pyridoxine would seem to indicate that the primary action must be on the release mechanism. However, the possibility of a direct stimulation of the myelocytic elements of the bone marrow is a question which can only be answered by extensive bone marrow studies.

Summary. Our results would seem to indicate that pyridoxine can bring about a rapid and significant rise in the number of circulating granulocytes in the blood in human subjects after a depression caused by thiouracil. Although there is a large element of self recovery after removal of the toxic agent there seems to be no doubt that the recovery process is much accelerated by pyridoxine.

15132

Experimental Tumors after Nerve Section in an Insect.*

BERTA SCHARRER. (Introduced by Ernst Scharrer.)

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In the course of a study of the endocrine functions of the corpora cardiaca and allata in insects the occurrence of tumors was noted. The corpora cardiaca in the species used (*Leucophaea maderae,* Orthoptera) are elongate structures behind the brain which continue postero-laterally into the corpora allata. In a series of experiments both corpora allata together with the posterior part of the corpora cardiaca were removed (Fig. 3, b). Following these operations tumors developed predominantly in 2 locations, namely, in the anterior portion of the alimentary canal and in the salivary reservoir. In rarer instances the salivary glands were involved (Fig. 1).

The appearance of these tumors was first suspected to be due to an endocrine disturbance resulting from the removal of the corpora allata or cardiaca. Such an interpretation would be supported by the fact that in young nymphs of another Orthopteran (Dixippus) allatectomy was followed by abnormal tissue growth and degeneration.[1] Furthermore, endocrine factors play a role in the origin of certain mammalian tumors.[2,3] Of the two endocrine glands here under consideration the corpora allata seemed by far more likely to be connected with the formation of neoplasms, since tumors developed also in such operations where a major portion or almost all of the corpus cardiacum tissue had been left intact (Fig. 3, c). Furthermore, the known hormonal actions of the corpora allata include the regulation of the rate of tissue growth and maturation, metabolism, and egg development.[4-8]

If the tumors to be described are caused by the lack of a corpus allatum hormone, it should be possible to prevent their occurrence by reimplantation of corpora allata into allatec-

* Aided by a grant from the Rockefeller Foundation.

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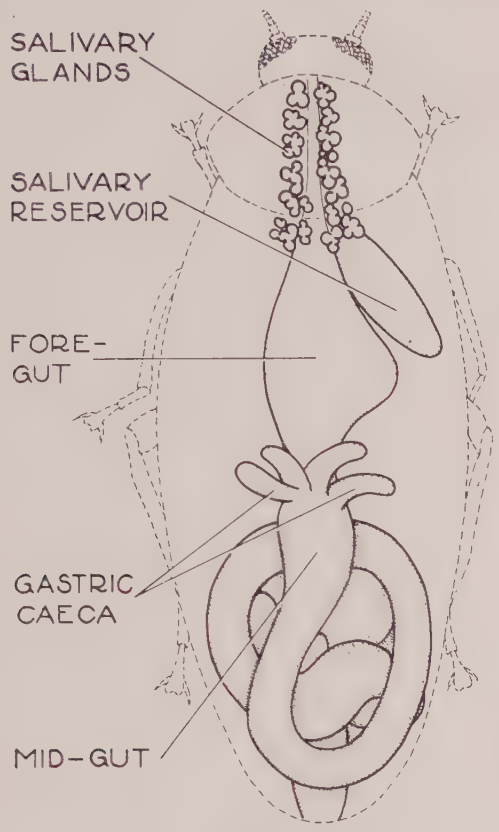

FIG. 1.

Diagram of *Leucophaea maderæ*. The organs indicated are sites of tumor formation. × 2¼.

tomized animals. This type of operation was performed in 231 male and female adults with, however, no apparent effect on the incidence of tumors. At least a certain percentage of these implants must have taken because egg development occurred in 56 females of this group. Tumors were also found after unilateral allatectomy (49 cases). This operation, however, did not prevent normal egg development which fact shows that one corpus allatum can fulfill the endocrine function of both glands.

These results suggested that the extirpation of the corpora allata may not have been the cause of tumor formation. The destruction of or interference with the normal function of some neighboring structure occurring accidentally during allatectomy was, therefore, suspected. Consequently pains were taken to improve the technic of allatectomy so that damage to neighboring structures was reduced as much as possible. The incidence of tumors after these improved operations decreased appreciably. The question arose which structures in the immediate proximity of the corpora allata and cardiaca could be involved.

Of such structures the recurrent nerve was given special attention, because its distribution, as traced in methylene blue preparations, showed a relationship to the sites of tumor formation.

The recurrent nerve belongs to the stomatogastric, *i.e.*, sympathetic nervous system, and takes its origin from the frontal ganglion (Fig. 2, 3). After passing between brain and pharynx the nerve becomes most intimately connected with the corpora cardiaca. Here is located a second sympathetic ganglion (hypocerebral ganglion) which in Leucophaea is represented by an inconspicuous group of

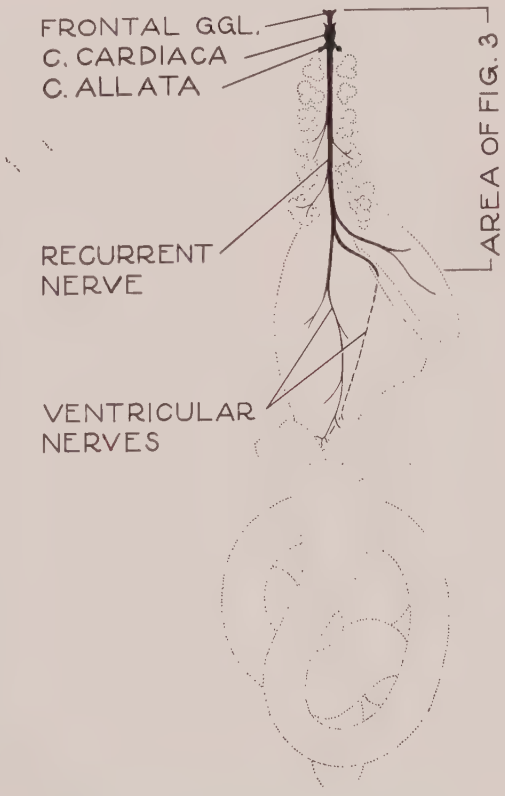

FIG. 2.

Diagram of innervation of salivary glands, salivary reservoir, and anterior portion of alimentary canal by branches of the recurrent nerve. × 2¼.

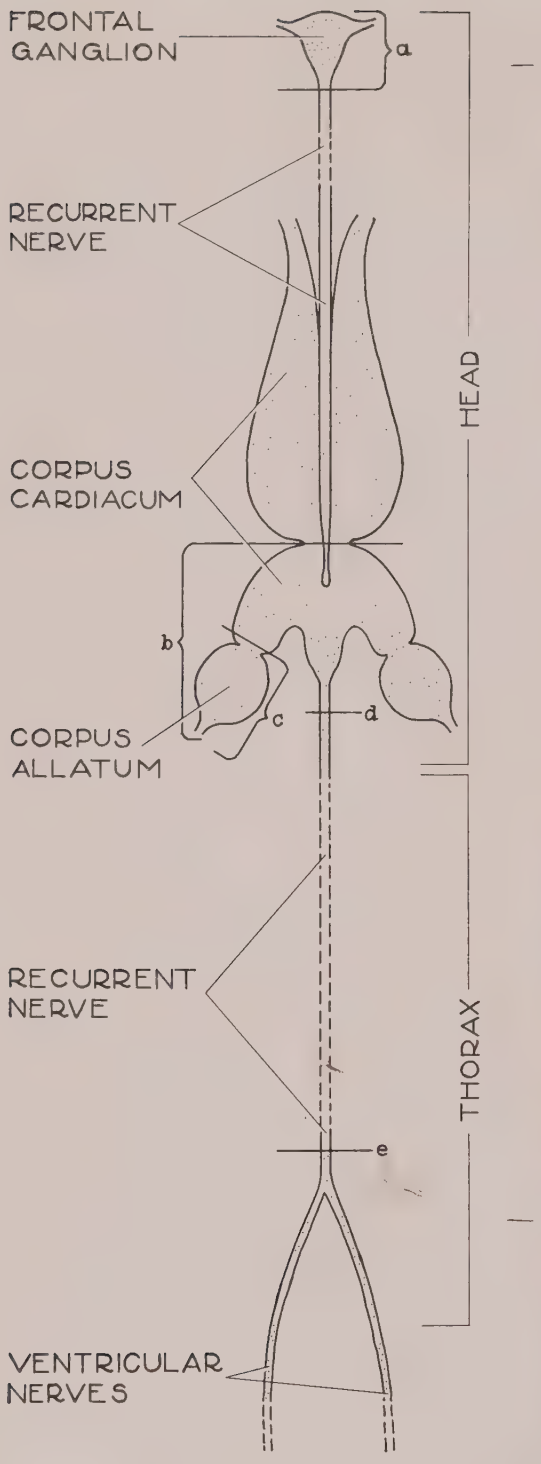

FIG. 3.
Diagram of stomatogastric nervous system and corpora cardiaca and allata of *Leucophaea maderæ*. a-e indicate the following operations: a, Extirpation of frontal ganglion. b, Removal of corpora allata and posterior corpora cardiaca. c, Allatectomy. d, Section of recurrent nerve close to corpora cardiaca. e, Section of recurrent nerve near bifurcation.

FIG. 4.
Tumor in wall of salivary reservoir. × 35.

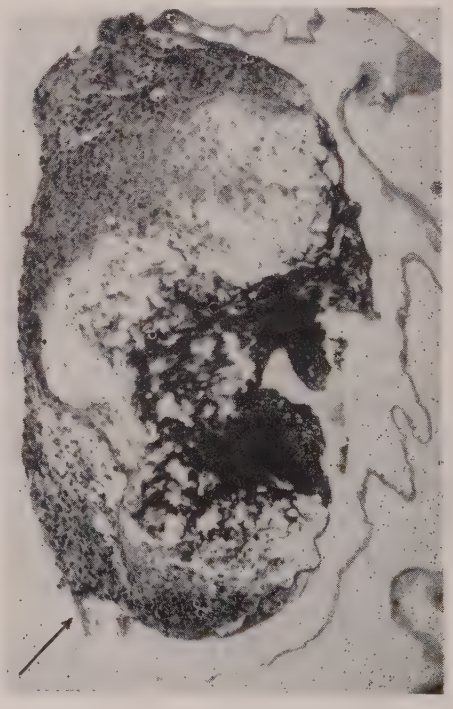

FIG. 5.
Tumor in wall of salivary reservoir. The border between the thin wall of the salivary reservoir and the tumor is well marked (arrow). Three layers can be distinguished. An outer layer consists of small cells, a middle layer of swollen, vesiculated cells, an inner layer facing the lumen of the reservoir consists of brown cell debris. × 80.

ganglion cells associated with the recurrent nerve. In the thoracic region the recurrent nerve divides into 2 rami (ventricular nerves), and gives off branches to the salivary glands and to the wall of the salivary reservoir. The ventricular nerves continue to follow the alimentary canal which they innervate (Fig. 2). In Leucophaea they could be traced as far caudad as the gastric caeca which mark the beginning of the mid-gut (ventriculus). In this region the nerves ramify into thin branches whose actual termination could not be ascertained.

From this description it is obvious that during an operation in the region of the corpora cardiaca and allata the recurrent nerve may easily be severed (Fig. 3, b, c). In fact, this was demonstrated to have occurred in a considerable number of cases available for post mortem dissection or histological study. There was good agreement between the incidence of nerve injury and tumor development. It seemed not unlikely, therefore, that the accidental cutting of the recurrent nerve during cardiac- and allatectomy was the cause of the formation of tumors.

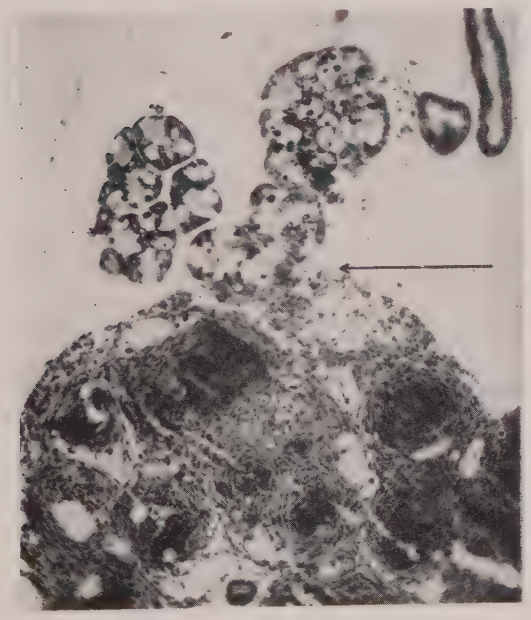

FIG. 6.
Tumor in salivary gland. Normal glandular tissue and salivary ducts (at right) above arrow. Tumor mass with remnants of glandular tissue beneath arrow. The tumor cells show whorl formation with degeneration in center. × 80.

This possibility was investigated in 3 series of experiments.

(1) The recurrent nerve was severed in 42 males and 3 females behind the corpora cardiaca and allata, care being taken to leave the glandular complex itself intact (Fig. 3, d).

(2) The recurrent nerve or its 2 ventricular branches were cut in 9 males in the thoracic region, *i.e.*, in a region considerably distant from the corpora cardiaca and allata (Fig. 3, e).

(3) The frontal ganglion which contains part of the cells of origin of the recurrent nerve was extirpated in 36 males, 14 females, and 13 nymphs (Fig. 3, a).

All 3 types of operation resulted in tumor formation. In series 1 and 2 the incidence was particularly high (approximately 80%). This seems to furnish ample proof that the neoplasms observed in the anterior portion of the alimentary canal and in the salivary complex were caused by the interference with their innervation rather than by a disturbance of the endocrine balance.

A more detailed description of the mode of growth and of the histological characteristics of the tumors here under consideration will be given in another paper. For the present it may suffice to say that they are well defined conspicuous tissue masses which in advanced stages can be easily noticed with the naked eye.

In histological preparations the borderline between the normal tissue and the tumor is usually well marked. The tumors consist of layers of cells whose appearance becomes progressively abnormal as the tumor enlarges. The cells of consecutive layers differ in various respects. In one layer, for instance, a considerable percentage of nuclei may be pycnotic, while in another they may be irregularly vesicular containing little chromatin. Often, near the lumen, these cells break down into a debris of brownish color (Fig. 5). This description applies only in a general way to the various tumors mentioned. Differences are observed in accordance with the tissues in which the tumors develop as, for instance, in salivary glands (Fig. 6) or fore-gut (Fig. 7).

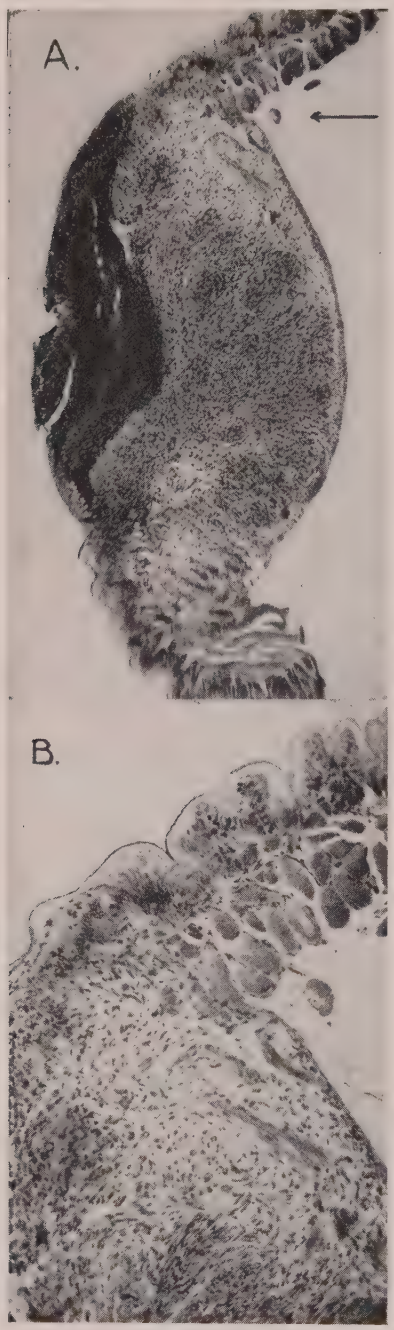

FIG. 7.

A. Tumor in wall of fore-gut. Above and beneath tumor the wall is normal consisting of a chitin-covered epithelium facing the lumen (to the left), and a muscularis. The stratification of the tumor is pronounced. × 35.

B. The tumorous transformation at the upper end (arrow) is shown at higher power. All components of the fore-gut wall are involved. × 80.

The part of the alimentary canal which is most commonly the site of extensive tumorous growth following the cutting of the recurrent nerve is the anterior portion of the mid-gut. At an early stage a ring-shaped thickening of the ventricular wall may be observed in the region of the gastric caeca. From there the neoplastic growth appears to proceed posteriorly for some distance. The intestinal wall, normally thin and more or less transparent, becomes thick and opaque. The columnar epithelium as well as part of the muscularis is replaced by multiple layers of cells. Examples are illustrated in Fig. 8 and 9. Fig. 8 shows the transition of the normal columnar epithelium of the mid-gut (left of arrow) into the irregular, multilayered tumor mass (above arrow). Fig. 9A illustrates the borderline between the epithelium of the esophageal valve (left of arrow) and the normal mid-gut epithelium (right of arrow) in an unoperated animal. In Fig. 9B a corresponding section through the alimentary canal of an animal in which the recurrent nerve had been cut is shown. The epithelium of the esophageal valve is unchanged, whereas the mid-gut epithelium is replaced by strati-

FIG. 8.

Mid-gut tumor showing transition (arrow) from normal columnar epithelium to multilayered tumor mass, overlapping normal epithelium. × 80.

FIG. 9.

A. Intestinal wall at transition from esophageal valve (left of arrow) to mid-gut (right of arrow) in normal animal.

B. Corresponding section from an operated animal. Esophageal valve unchanged (left of arrow); tumor in mid-gut wall (right of arrow). × 80.

fied tumor tissue (right of arrow). Similar tissue changes as those described for the mid-gut may also be observed in the wall of the fore-gut (Fig. 7 A, B), but are, as a rule, less extensive and are less frequently encountered.

The severity of the tissue transformation makes it appear unlikely that the normal functions of the anterior portion of the alimentary canal can be maintained. In fact, animals with tumors of this kind died at intervals from 10 days to several months following the nerve lesion. The anterior portion of the alimentary canal was frequently filled with an abnormally large quantity of food, while at the same time the fat body was depleted. Death thus seems to be due to starvation.

Tumors growing from the thin, normally transparent wall of the salivary reservoir are conspicuous (Fig. 4 and 5). They may be round, oval, or irregularly shaped. The salivary reservoir may contain one large tumor (up to 10 mm in diameter) or several smaller ones. They consist of a thick layer of opaque tissue which, similar to that encountered in the mid-gut, appears to become gradually broken down into brown debris (Fig. 5).

The experimental tumors described were obtained in nymphs as well as in male and female adults ranging, at the time of operation, from shortly after emergence to 10 months of adult age. Altogether over 300 cases with tumors were studied.

In the literature reports on the occurrence of tumors in insects are rare. Brun[9] described a brain tumor in an ant, Stark[10–12] hereditary tumors in Drosophila. In view of the experimental findings in Leucophaea a search for spontaneously occurring tumors in this species was made. Among hundreds of unoperated animals, dissected at various ages, none had tumors in the salivary complex. However, a few specimens showed changes in the alimentary canal which, although of milder degree, were comparable to the tumors developing after the section of the recurrent nerve. Whether or not these tumors of the "normal" insect may be caused by some damage to the stomatogastric nervous system, or by irritation due to intestinal parasites cannot be decided.

Conclusion. (1) An insect is introduced as a suitable experimental animal offering new possibilities for the study of tumors.

(2) Interruption of a nerve has been shown to cause tumorous growth in organs which it supplies. Further experiments, already under way, must show whether this phenomenon occurs only in insects or applies also to vertebrates.

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15133

Alloxan Diabetes in Rabbits.* Production of Hypercholesterolemia, Hyperlipemia and Adrenal Cortical Lesions.

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Dunn and his coworkers[1] reported that a single intravenous injection of alloxan into rabbits caused extensive damage to pancreatic islet cells and suggested that it should lead to the production of sustained diabetes mellitus. Alloxan diabetes has since been produced in the rabbit, rat, dog, cat, rhesus monkey, pigeon, and guinea pig.[2,3,4] Hyperlipemia and hypercholesterolemia are commonly found in patients with diabetes mellitus. However, up to the present, few reports have been made of the effect of alloxan upon the serum lipids of experimental animals. Bailey and Bailey[5] reported that one rabbit with uncontrolled diabetes following an intravenous injection of 200 mg of alloxan per kilo of body weight, developed pronounced hyperlipemia with total blood lipids of 8412 mg per 100 ml. The hyperlipemia was not observed in other rabbits in which the diabetes was controlled with insulin. Goldner and Gomori[6] found that alloxan diabetes in dogs was characterized by hyperlipemia and fatty liver which developed within 10 to 12 days. A more extensive study of the effect of alloxan upon the blood lipids of rabbits is reported in this paper.

Experimental. Thirty-eight rabbits varying in age between 5 months and 3 years were used. The rabbits were kept in individual cages and fed a diet of Purina Rabbit Chow and oats. Fresh greens were given twice a week. Fresh drinking water was supplied daily. Without a preliminary fasting period each animal was given a single intravenous injection of a freshly prepared 5% solution of alloxan monohydrate (Eastman Kodak Co.). Three rabbits were given a dosage of 50 mg of alloxan per kilo of body weight, 3 a dosage of 100 mg/kg, and the remaining 32 received a dosage of 125 mg/kg. The hypoglycemic shock which frequently follows alloxan injection was controlled in most cases by subsequent intravenous injection of 1 ml of 50% glucose per kilo of body weight after 6 hours, and by substituting a 5% glucose solution for the drinking water during the first 24 hours. Blood samples were taken for analysis at various intervals. Blood sugar was determined by the colorimetric copper method of Benedict,[7] and total cholesterol by the Sperry-Brand method.[8] The total lipids were determined gravimetrically by a method shortly to be described. Autopsies were done on all animals dying during the experiment and histological examination was made of all tissues.

Results. Alloxan Dosage 50 mg/kg: The 3 rabbits of this group all developed hyperglycemia within 3 hours of the injection. No hypoglycemic phase was observed in samples taken at 6 and 24 hours. The blood sugar of 2 of the rabbits returned to normal within 4 days; that of the third remained elevated above 200 mg % for 60 days and then gradually fell to 130 mg % 6 months after the injection of alloxan. At no time were serum lipid or cholesterol values found above basal

* This investigation has been aided by a grant from the Albert and Mary Lasker Foundation.

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levels. One rabbit with normal blood sugar died 112 days after alloxan injection. No lesions referable to alloxan were found. The other 2 animals were still living 176 days after injection.

Alloxan Dosage 100 mg/kg: All 3 rabbits of this group developed diabetes. Two of the 3 have survived 176 days since the injection. The blood sugar of one of these rabbits has remained above 300 mg % during the whole period; that of the other was above 300 mg % for 20 days and then gradually fell to normal values. The serum cholesterol and lipid values of both animals have remained within normal limits during the entire period. The third rabbit died on the 4th day after injection. At that time the blood sugar was 490 mg % and the serum cholesterol 520 mg %. The serum was extremely milky. At autopsy, in addition to the expected damage to the pancreas, the animal showed extensive fatty infiltration of the liver and kidneys and marked necrosis and leucocytic infiltration of the cortex of both adrenal glands. No alterations were visible in the adrenal medulla.

Alloxan Dosage 125 mg/kg. The triphasic nature of the blood sugar response was studied in 3 rabbits to which no glucose was given after the injection of alloxan. Samples of blood were taken hourly for 10 hours following injection and at daily intervals thereafter. Blood sugar levels rose from normal (98, 92, 90 mg %) to hyperglycemic levels (416, 296, 436 mg %) in from 3 to 4 hours and then fell to a low level (68, 22, 17 mg %) 8 to 10 hours after injection. At 24 hours the values were above normal (122, 234, 166 mg %) and one day later hyperglycemic levels were found (372, 400, 340 mg %). One rabbit died on the 4th day after injection, one on the 5th, and one has survived 60 days without a decrease in the blood sugar. All 3 animals had developed hypercholesterolemia (200, 333, 410 mg %) and hyperlipemia (1.5, 4.6, 6.3 g %) 4 days after injection. Morphological examination of the tissues of the rabbit dying on the fourth day revealed no lesions except loss of the beta islet cells of the pancreas. The rabbit dying on the 5th day showed necrosis of the renal convoluted tubules in addition to the pancreatic lesion.

Of the remaining 29 rabbits of this group, all of which received glucose, two-thirds died within 5 days, and all but 7 died within 60 days following the injection.

Determinations of blood sugar and serum cholesterol were made on all (25) animals surviving 4 days or longer. Total serum lipids were determined on 11 animals. Of the 7 rabbits surviving more than 60 days 2 were resistant to alloxan and showed normal values for blood sugar, cholesterol, and total lipid throughout the entire period. One developed hyperglycemia (blood sugar values in excess of 300 mg % throughout the entire period) without any elevation in cholesterol or total lipids, while 4 showed elevation of all 3 substances. Details of the data on 3 of the surviving rabbits are shown in Fig. 1. Two other survivors with hyperglycemia gave a pattern similar to that of the second.

All of the animals that died later than 4 days following the injection developed hyperglycemia (blood sugar >300 mg %), hypercholesterolemia (values ranging between 200 and 780 mg %) and hyperlipemia (values ranging between 2.8 and 18.5 g %). Fig. 2 shows the data on 8 of the 13 animals in this group. Since but one analysis was obtained for each of the other five animals, they are not included in the figure. They all showed cholesterol values above 200 mg % and the sera were visibly lipemic.

Post Mortem Findings. Rabbits dying after 3 days had a weight loss of from 10 to 30%, associated with considerable decrease in fat in subcutaneous, omental, and intraperitoneal tissues. After the 4th day livers were decreased in size, were dark brown and had sharp edges. In several instances the adrenal glands were congested and some showed greenish yellow mottling in the cortex. The kidneys of many of the rabbits were pale, swollen, and tan colored. Their capsules were easily stripped and their widened cortices revealed gray and yellow streaks running parallel to the tubular striations.

Microscopically, aberrations from normal similar to those described by other investiga-

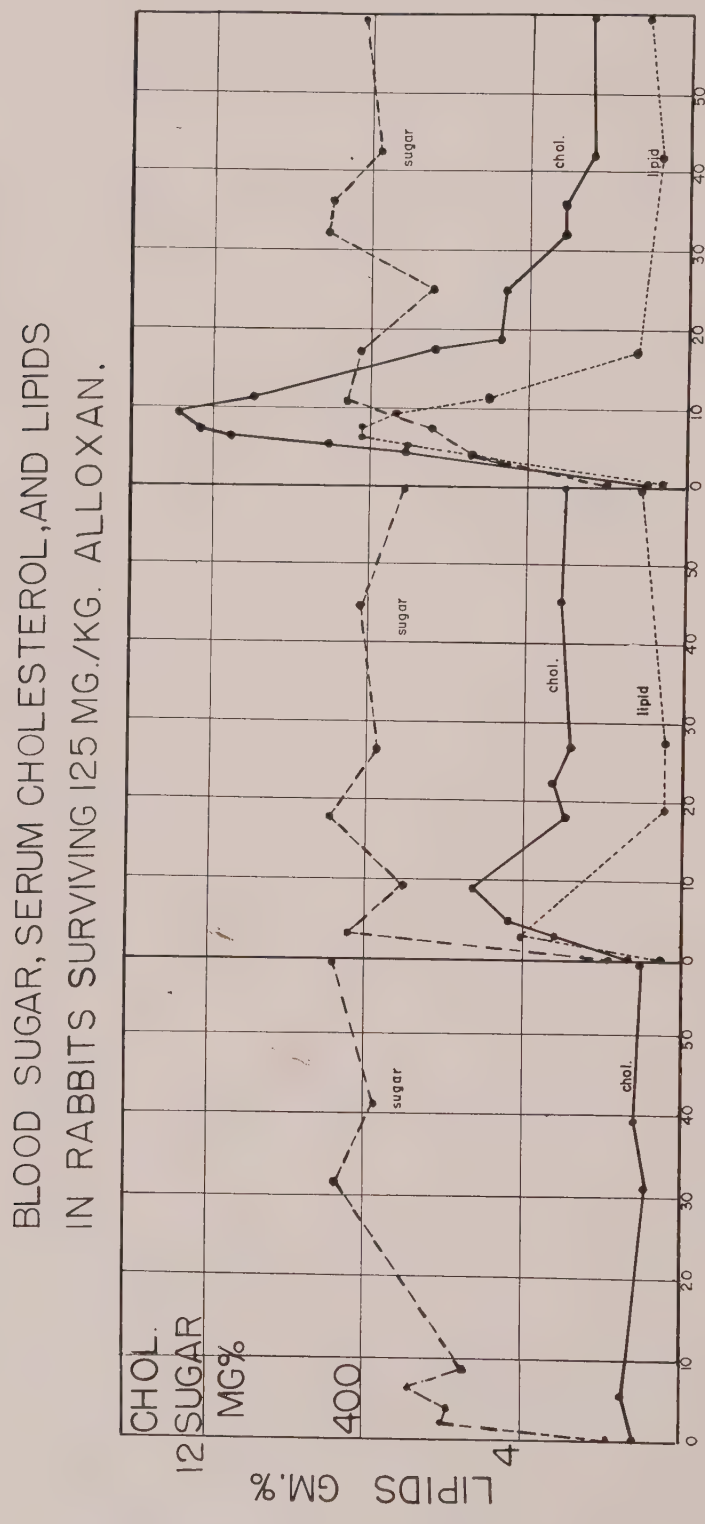
BLOOD SUGAR, SERUM CHOLESTEROL, AND LIPIDS
IN RABBITS SURVIVING 125 MG./KG. ALLOXAN.
CHOL.
SUGAR
MG%
400
12
4
LIPIDS GM.%
sugar
chol.
lipid
TIME IN DAYS
0
10
20
30
40
50

FIG. 1.

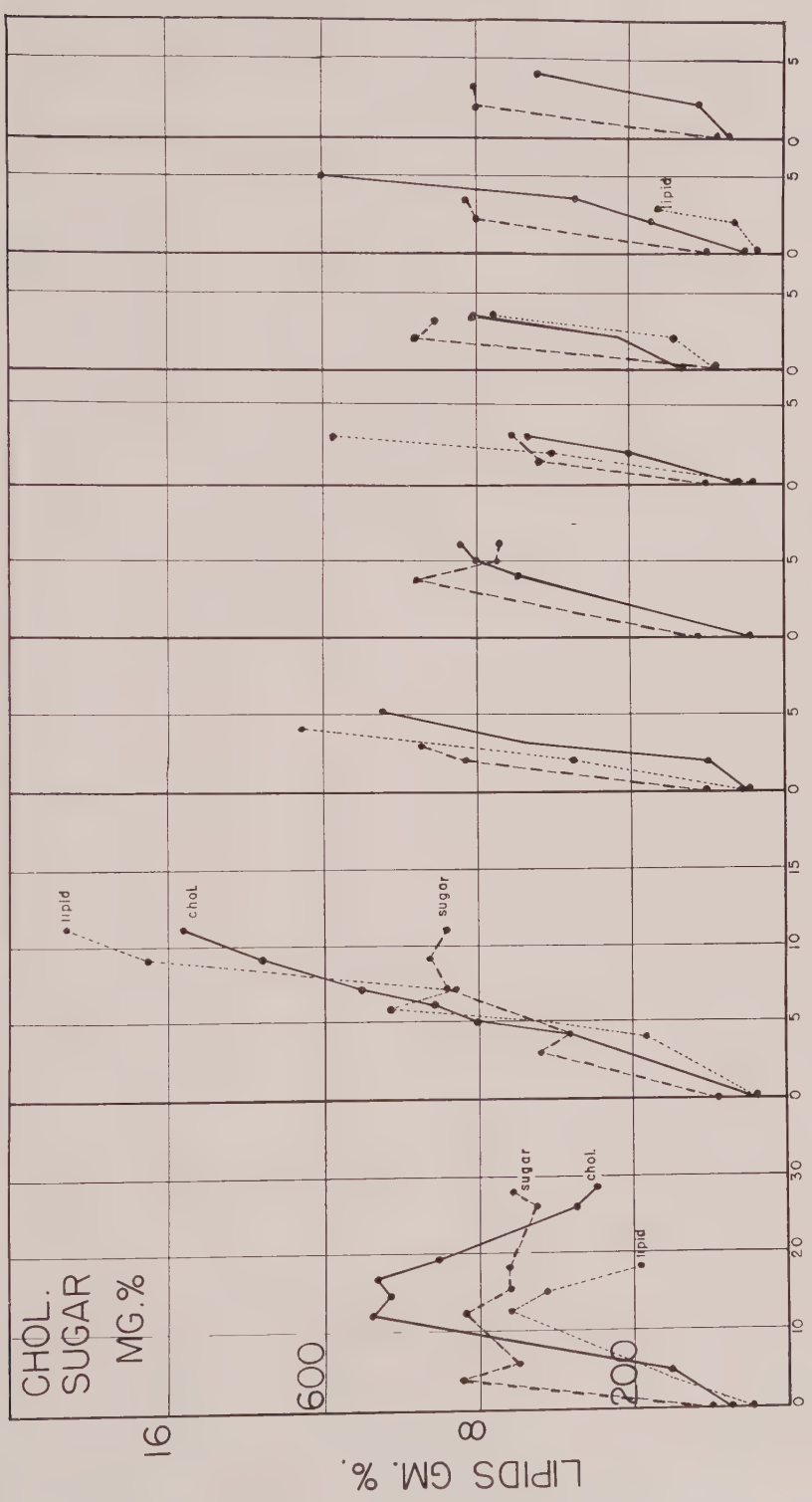
BLOOD SUGAR, SERUM CHOLESTEROL, AND LIPIDS
IN RABBITS DYING AFTER 125MG./KG. ALLOXAN.
CHOL.
SUGAR
MG.%
600
200
16
8
LIPIDS GM. %.
lipid
chol.
sugar
TIME IN DAYS

FIG. 2.

FIG. 3.
Adrenal Cortex rabbit No. 354, four days after i.v. injection of 125 mg/kg of alloxan. Necrosis of cortical cells and infiltration with polymorphonuclear, neutrophilic, and eosinophilic leucocytes. Magnification 100 ×. Giemsa stain.

tors were found in the pancreas,[9,10] liver,[6] and kidneys.[9] Lysis of beta cells of the islets of Langerhans was observed in all rabbits dying within 3 days after alloxan administration. Thereafter the islets consisted of eosinophilic alpha cells surrounding the collapsed stroma which previously had supported beta cells. Liver cells were well vacuolated until the 4th day. After that time they lost their vacuoles and their cytoplasm became more granular and denser than normal. Necrosis and regeneration of epithelium of renal convoluted tubules occurred bilaterally in 9 of 23 rabbits. Hyalin casts and, in one rabbit, calcium containing casts were seen in the damaged tubules.

Lesions of a type not previously described in alloxan poisoning were found in the cortices of both adrenal glands of 12 of 25 animals. Diffuse and focal necrosis was encountered bilaterally in the adrenal cortices of 7 of these animals. The necrosis was accompanied by infiltration with many polymorphonuclear leucocytes together with a few mononuclear and eosinophilic cells (Fig. 3). Bacterial stains, such as Giemsa or Brown, revealed no organisms in these areas. In 4 cases loss of lipoid and decrease in size of cortical cells was seen. One rabbit lived 32 days after alloxan treatment and showed considerable focal lymphocytic infiltration in addition to cell atrophy in the adrenal cortex. In contrast with the findings of Hard and Carr[10] no adrenal medullary changes were observed.

Discussion. It is evident that uncontrolled diabetes mellitus produced in rabbits by the intravenous injection of alloxan may be accompanied by a great increase in serum cholesterol and lipids. Hypercholesterolemia and hyperlipemia always occurred if the diabetes was severe enough to cause the death of the animal. Death after the fourth day is attributed to the diabetic condition rather than to the acute effects of alloxan. If the animal survived, great variations occurred in the degree of hyperlipemia and hypercholesterolemia, as shown in Fig. 1 for 3 rabbits all of which had diabetes of comparable severity as meas-

[9] Dunn, J. S., Kirkpatrick, J., McLetchie, N. G. B., and Telfer, S. V., *J. Path. and Bact.*, 1943, **55**, 245.

[10] Hard, W. L., and Carr, C. J., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 214.

ured by blood sugar levels. In the animals that died, no correlation could be found between the occurrence of lesions in the adrenal cortex or the kidneys and the development of high serum levels of cholesterol and lipid. In view of the results reported by Bailey, Bailey, and Leech[11] on rabbits and by Goldner and Gomori[6] on dogs, the hyperlipemia is probably caused by the diabetes itself. They found that it did not occur if the hyperglycemia was controlled with insulin. The type of curve found for these values in surviving animals together with the observation made upon the animals that died, suggests that the high levels were caused by a flood of fat being mobilized from the body depots. The mechanism of this fat mobilization will be studied further.

Age seemed to play some role in the reaction of the rabbits to alloxan. Of 32 rabbits given 125 mg/kg of alloxan, 17 were 5 months old and 15 of ages varying between 9 months and 3 years. Six of the 17 young rabbits, one of which was resistant to alloxan, survived, whereas only one old rabbit out of 15 survived and that one was resistant to alloxan. Only 2 of the 17 young rabbits died before the 5th day as compared with 9 of the 15 older ones. The younger group was remarkably uniform in that 7 of the 11 fatalities occurred on the 5th day. Only 4 of the 11 young rabbits that died showed extrapancreatic lesions, whereas only 2 of the 14 in the older group failed to show such lesions. Necrosis of the adrenal cortex was found only in the older animals. Loss of vacuolization of the cells of the adrenal cortex occurred in both age groups. An adequate number of control animals in the old age group were not available for study at this time. However, during the past five years the adrenals of 279 rabbits in this age group have been examined without disclosing any comparable lesions. Moreover, the acute nature of the lesion relates it definitely to the alloxan injection rather than to an unknown factor referable to the age of the animal. This demonstration of damage to the adrenal cortex is of particular interest in view of the findings of Kirschbaum, Wells, and Molander[12] that the adrenal cortex is involved in the initial hyper- and hypoglycemic reactions to alloxan.

Summary. A transient period of hypercholesterolemia and hyperlipemia may accompany the early stage of severe diabetes mellitus in rabbits following the injection of alloxan. It occurred in varying degrees in many rabbits that survived with persistent diabetes and always occurred if the diabetes was severe enough to cause death. A dosage of 50 mg/kg of alloxan failed to cause hyperlipemia or hypercholesterolemia, 100 mg/kg produced it in one of 3 rabbits, while 125 mg/kg was effective in 29 of 32 animals. Lesions were found in the adrenal cortices of 12 of 25 animals dying after receiving 125 mg/kg of alloxan.

11 Bailey, C. C., Bailey, O. T., and Leech, R. S., *New Eng. J. Med.*, 1944, **230**, 533.

12 Kirschbaum, A., Wells, L. J., and Molander, D., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 294.

15134

Horse Brain Thromboplastin: II. Effect of Various Factors on Activity of Horse Brain Extracts.*†

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It has been suggested[1] that horse brain thromboplastin might be used as a reagent for the quantitative determination of prothrombin by the one-stage method of Quick.[2] The optimal conditions necessary for the preparation of the reagent solution from acetone-dried horse brain powder have not previously been studied; heretofore, the concentration, temperature, and period of activation employed were the same as those used by Quick for rabbit brain thromboplastin. The influence of these factors on the activity of extracts prepared from desiccated horse brain has now been investigated. In addition, a modified technic for the preparation of the acetone-dried horse brain powder has been evolved.

Experimental Results and Discussion. A. Preparation and activity of acetone-dried horse brain powder. The desiccated horse brain powder used in these experiments was prepared by acetone dehydration of fresh brains.‡ The basic principle of this method does not differ from that previously used for horse brains[1] nor from the method of Quick for rabbit brain thromboplastin.[2] However, the technic employed is different. In the present procedure a stainless steel press was employed for the removal of the pia mater and the major portion of the blood vessels, and a colloid mill was used for the rapid acetone desiccation of the remainder of the brain tissue. Approximately 11% of the tissue failed to pass through the press and was discarded. This procedure permitted the handling of whole horse brains with a minimum of time and effort. It was possible to suspend each horse brain in acetone within 20 to 30 minutes from the time it was obtained from the animal. With this rapid and convenient means for desiccating brain tissue, relatively large quantities of material, *i.e.*, 300 g or more, could be prepared in a single day. Furthermore, the new method has the advantage of producing a product with a relatively constant thromboplastic activity, which was not true of the previous method. The yields and the activities of several batches of brain powder are reported in Table I.

In determining thromboplastic activity, 6% suspensions of brain powder were prepared in 0.85% sodium chloride solution that had been heated previously to 50°C, and the suspensions were held at this temperature for 15 minutes, while stirring at intervals with a glass rod to break up as many as possible of the large particles of powder. The suspensions then were centrifuged at approximately 3000 r.p.m. for 30 seconds, just long enough to remove the coarse particles. (The concentration of solids remaining in the supernatant was approximately 2%.)

The activities of the saline extracts and solutions prepared from these extracts were determined by the one-stage method of Quick,[2] using citrated human plasma and 0.184% calcium chloride solution. A concentration of 0.184% calcium chloride was optimal for our standard plasma.[1] Except where indicated, all determinations were made on one sample of pooled (10 bleedings) citrated human plasma (prepared from blood containing 50 cc of 4% sodium citrate in each 500 cc). Ten

* Kazal, L. A., and Arnow, L. E., *Arch. Biochem.*, 1944, **4**, 185, is No. I in this series.

† Preliminary report appeared in *Am. Chem. Soc. Abstracts*, Sept. 11, 1944.

1 Kazal, L. A., and Arnow, L. E., *Arch. Biochem.*, 1944, **4**, 183.

2 Quick, A. J., *J. Am. Med. Assn.*, 1938, **110**, 1658; 1938, **111**, 1775.

‡ We are very grateful to Dr. B. J. McGroarty for kindly arranging for the collection of horse brains, and to Mr. G. Roberts for his cooperation in the preparation of the brain powder.

TABLE I.
Horse Brain Thromboplastin Preparations.

Preparation No.	No. of brains	Wet wt whole brain, g	Wet wt pressed brain, g	Acetone-dried brain powder, g	Activity* clotting time, (sec)
1	4	2025	—	324	16.1
2	4	2190	1940	382	16.1
3	5	2820	2500	455	15.8
4	6	—	2690	490	16.2
5	6	—	2960	538	15.4
6	5	—	2490	464	15.8

* Clotting time of 0.1 cc of a standard citrated human plasma recalcified with 0.1 cc of 0.184% calcium chloride solution in the presence of 0.1 cc of thromboplastic extract that had been diluted with an equal volume of distilled water.

cc portions of this plasma were frozen rapidly in small glass vials in a methyl "cellosolve"-dry ice mixture, and were stored at –20°C. Samples were thawed at 37°C in a water bath just before use. The clotting time of this plasma remained constant throughout the period of investigation. All clotting time determinations reported are the average of 3 to 5 individual readings.

In order to determine whether or not optimal conditions were being employed for the preparation of these extracts, a study was made of 4 variables in the procedure, namely, concentration, method of extraction, temperature of extraction, and the duration of heat treatment.

B. *Effect of concentration of brain powder on thromboplastic activity.* Brain powder suspensions ranging in concentration from 1% to 20% were prepared in physiological saline solution at 50°C and centrifuged as described above. The supernatants were diluted with distilled water, except in the case of the 20% suspension of which only enough could be obtained for a few tests. The activities of each extract and its dilutions are presented in Table II (non-homogenized preparations). The most active extract was obtained from a 6% suspension of brain powder. Dilution of this extract with an equal volume of water did not significantly change its activity. Very often, however, such dilution will effect an increase of thromboplastic activity.[1] An example of this may be found in Table III, preparation No. 2.

Attempts to enhance the thromboplastic activity of brain powder suspension by increasing the amount of solids in suspension by homogenization were unsuccessful. Suspensions (1% to 20%) were prepared as above with the exception that, after the 50°C incubation, they were homogenized in a water-cooled colloid mill for 1 to 2 minutes instead of being centrifuged. These emulsions were tested for activity in the same manner as the non-homogenized preparations. The results,

TABLE II.
Effect of Concentration on Thromboplastic Activity of Homogenized and Non-homogenized Saline Extracts of Acetone-dried Horse Brain Powder.

Preparation	Conc. of brain powder susp., %	Dilutions with distilled water: 100% (undiluted)	50%	25%	10%	1%
		Plasma clotting times (sec.)				
Non-homogenized	3	18.2	23.8	33.4		
	6	**16.1**	16.7	19.7		
	10	17.8	18.0	22.4		
	20	32.5				
Homogenized	1	32.0	28.5	30.6	34.4	
	3	36.6	28.0	28.2	28.5	51.0
	6	66.6	39.0	29.8	**27.7**	29.5
	20	152.5	87.0	43.7	29.5	37.0

Numbers in heavy type are the minimal clotting times for each type of preparation.

TABLE III.
Influence of Method of Extraction on Thromboplastic Activity of Saline Extracts of Acetone-Dried Brain Powder.

Methods of extraction (6% susp. of brain powder)	Dilutions with distilled water		
	100% (undiluted)	50%	25%
	Plasma clotting times (sec.)		
1. No stirring	17.6	19.0	
2. Mild stirring (manually)	19.2	16.8	19.4
3. Vigorous stirring (air-driven stirrer)	22.1	21.6	23.2
4. Homogenization (colloid mill)	36.6	28.0	28.2

which are presented in the second section of Table II, show that all such preparations exhibited a lower order of thromboplastic activity.

C. *Influence of method of extraction.* The experiments on homogenization suggested that the activity of thromboplastic extracts could be influenced by the manner in which the extraction was made. Accordingly, saline extracts were prepared from 6% suspensions as described, except that during the period of incubation at 50°C, one extract (No. 1) was not stirred after the brain powder had been moistened, another (No. 2) was stirred with a glass rod as usual, and a third (No. 3) was stirred vigorously with an air-driven stirrer. All extracts were centrifuged, and the supernatants tested for activity at various dilutions. The results in Table III, which include those of homogenized 6% suspensions for the sake of comparison, indicate that the method of mild stirring produced the most active extract. Vigorous stirring and homogenization proved to be detrimental. When stirring was not employed, the resulting extracts were fairly active, but lacked the advantage of increased activity upon dilution which the mildly stirred extracts possessed. The ex-

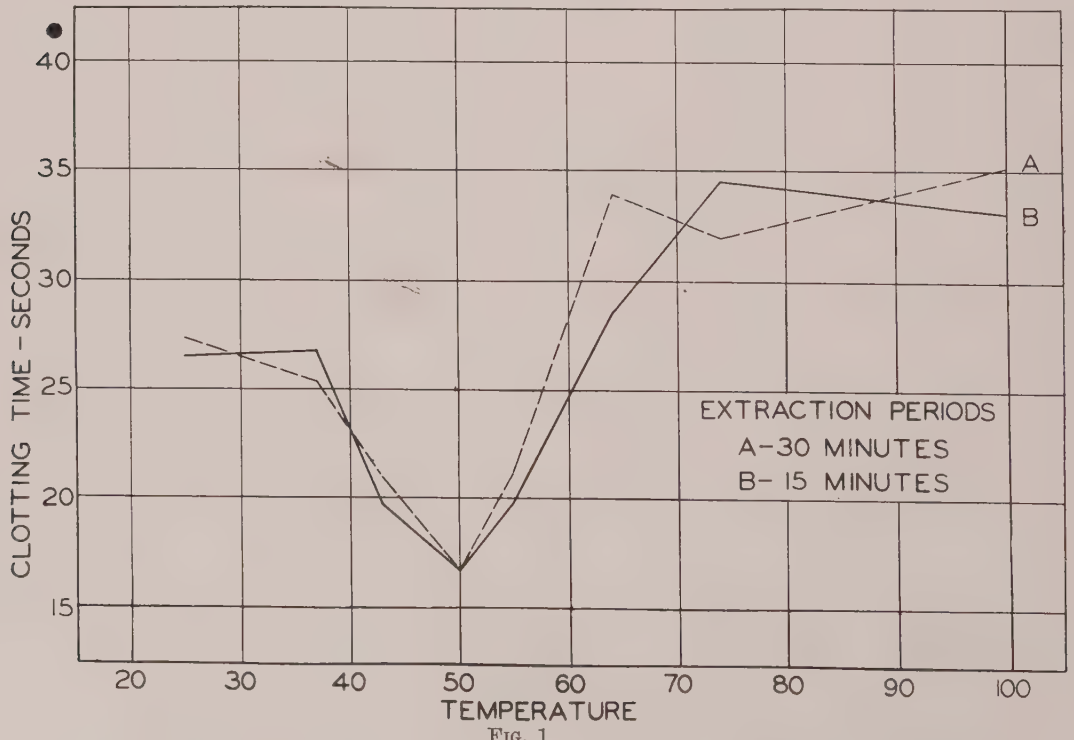

FIG. 1.
Effect of temperature on thromboplastic activity of suspensions of a horse brain powder.

periments on the effect of concentration and methods of extraction suggest the presence of a blood clotting inhibitor in horse brain, which is released under certain conditions.

D. *Effect of temperature and duration of extraction.* Temperatures ranging from 25°C to 100°C for periods of 15 to 30 minutes were substituted for the standard temperature of 50°C, and temperatures of 25°C and 50°C were investigated further over a period of 1 to 300 minutes. Except for these changes the technics of preparation and testing of the extracts were the same as those described in section A. The testing, however, was limited to the 50% dilutions of the supernatant. Fig. 1 illustrates the effects observed. A temperature of 50°C for a period or 15 to 30 minutes proved to be optimal. It appears that in the temperature range of 25° to 50°C an activation of the thromboplastic substance occurs. Also, at 50°C activation took place very rapidly; extracts exposed to this temperature for only a few minutes were considerably more active than similar extracts maintained at 25°C. In fact, at the latter temperature the thromboplastic activity at no time approached that of the extracts heated at 50°C although at both temperatures individual maximal activity was attained in 15 minutes.

In the Quick technic, a temperature of 50°C apparently was employed for the destruction of any prothrombin that might be present in the thromboplastic extract of rabbit brain.[3] Although destruction of prothrombin undoubtedly takes place under these conditions, it is believed that, at least for horse brain, the increased activity obtained after incubation at 50°C may be due to other factors as well. For example a centrifuged suspension of brain powder prepared at 30°C is less active than one prepared at 50°C. Significantly, the activity of the suspension prepared at the lower temperature cannot be increased by subsequently heating it at 50°C for as long as 25 minutes. On the other hand, when this suspension is heated at 50°C for 15-20 minutes in the presence of the insoluble brain residue previously removed by centrifugation, its activity partially is restored. It would appear from such experiments that thromboplastin is not completely extracted by saline from the brain powder at temperatures below 50°C. Whether this temperature causes an increase in the dispersibility of the active principle, or perhaps minimizes the extraction of an inhibitor, can only be conjectured at the present time. These observations are in agreement with the experiments of Astrup and Darling[4] who observed a 300% increase in the potency of thromboplastic extracts of lung heated at 40°C for 1 hour. In their opinion the increased potency was due "to a greater solubility of the active substance, probably by obtaining a greater degree of dispersion."

The preparation of Astrup and Darling was inactivated at 56°C. Partial inactivation of horse brain thromboplastin under the conditions of our experiments occurred at temperatures above 50°C. Between 75° and 100°C there was no further decrease of thromboplastic activity. Total loss of activity was not observed at these temperatures.

Summary. A modified procedure for the preparation of relatively large batches of acetone-desiccated horse brain powder has been developed. The thromboplastic activity was influenced by the concentration of brain powder used for the preparation of the saline extracts. The optimal mixture was a 6% suspension, whose supernatant quite often had optimal activity after dilution with distilled water. The optimal temperature for the preparation of saline extracts was 5°C; the optimal period of exposure to this temperature was 15 to 30 minutes. Exposure to temperatures between 50°C and 100°C for 15 to 30 minutes produced only a partial destruction of thromboplastic activity.

[3] Quick, A. J., *Am. J. Physiol.*, 1935, **114**, 282.

[4] Astrup, T., and Darling, S., *Acta. Physiol. Scand.*, 1942, **3**, 168.

15135

Prevention of Formation of End-Bulb Neuromata.*

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Painful amputation stumps become major problems during periods of extensive traumatic injuries such as result from modern warfare. Just why some amputation stumps become painful is not known. It has long been considered that end-bulb neuromas which form at the end of severed peripheral nerves may be the cause of this phenomenon, because these tumefactions may be painfully tender. All of these neuromas are not tender, and the excision of a tender neuroma does not always remedy a painful stump. The question has been raised as to whether the pain in an amputation stump is due entirely to the presence of a tender neuroma. If some procedure were devised by which neuroma formation could be prevented, the cause of painful amputation stumps could be more accurately determined. The complete elimination of neuroma formation in an amputation stump will be difficult because many small cutaneous nerves are divided and form neuromas, which may be potential sources of pain as proposed by Molotkoff.[1]

A recent review by Young[2] of the mechanism of regeneration of peripheral nerves, extensively studied by Cajal[3] and many others, indicates that the anatomico-biological characteristic of the peripheral nerve is one of unlimited capacity for growth of the regenerating axon so long as the central cell body of the neuron remains intact. This forward pressure of growth is maintained until the nerve fibers make functional connection with some type of end-organ, or under abnormal conditions, the fibers develop end-bulbs and Perroncito spirals,[3] making many types of peculiarly disorderly connections with the surrounding tissues typical of neuroma formation.[4] Recent evidence has shown the growth of the regenerating fibers under all conditions is guided by the "contact actions" of the surfaces along which they advance, thus returning in its essential implications to the "Mechanical Theory" of nervous regeneration originally propounded by Ranvier and Vanlair.[5] The growth, therefore, of nerve fibers to form a neuroma is a natural phenomenon. This growth is abnormal, because no sheath is present to prevent their irregular growth into scar tissue. The pain resulting from the manipulation of a neuroma may be due to the stimulation of these abnormal nerve endings by pressure or traction on the nerve fibers adherent to scar tissue. Boldrey[6] and Seddon[7] buried the nerve-end into bone and eliminated pain from the neuroma. Although a neuroma formed in the bone, it could not be stimulated mechanically.

Many methods have been devised in an attempt to prevent neuroma formation. These procedures can be listed under two groups: (1) those in which an attempt is made to kill or inhibit the growth of the nerve fibers by chemical and physical means, and (2) those which attempt to obstruct the pathway of the regenerating fibers mechanically. Recent reviews by Guttmann and Medawar[8] and Boldrey[6] discuss quite fully the different individual procedures. Huber and Lewis[4] originally suggested the injection of the stump

* Aided by a grant from Sharp and Dohme, Inc., Philadelphia.

1 Molotkoff, A. G., *J. Bone and Joint Surg.*, 1935, **17**, 419.

2 Young, J. Z., *Physiol. Rev.*, 1942, **22**, 318.

3 Cajal, S. R., *Degeneration and Regeneration of the Nervous System*, Oxford Univ. Press, 1928, **1**, 331 (Translated by R. M. May).

4 Huber, G. C., and Lewis, D., *Arch. Surg.*, 1920, **1**, 85.

5 Ranvier and Vanlair (cited by Cajal 3).

6 Boldrey, E., *Ann. Surg.*, 1943, **118**, 1052.

7 Seddon, H. J., 1941 personal communication, cited by Guttmann and Medawar 8.

8 Guttmann, L., and Medawar, P. B., *J. Neurol. and Psych.*, 1942, **5**, 130.

with 95% ethyl alcohol. Corner[9] advocated the excision of a wedge from the end of the nerve followed by suturing the two flaps, while Chapple[10] placed an epineural sleeve over the end of the nerve. Stookey[11] advocated a combination of the two procedures. More recently Kirk[12] and Bate[13] advocated electrodesiccation of the nerve stump. Following the simple ligation of the severed nerve with non-absorbable suture material sufficiently tightly to prevent regeneration of axones without rupturing the neurilemma sheath, Herrmann[14] reduced the occurrence of the phantom limb phenomenon to 5.8% in 120 patients subjected to amputation. Guttmann and Medawar[8] were unable to prevent consistently the regeneration of the axones of the sciatic nerve of the rabbit when using this procedure. In consideration of the mechanism of the regeneration of peripheral nerves, it becomes evident that no method which merely destroys an additional segment of nerve will result in prevention of neuroma formation. The neuroma will merely form at a higher level. Poth and Bravo Fernandez[15] in a preliminary report, discussed the implantation of the nerve stump into rigid tubes, closed at one end. This procedure quite effectively overcame the pressure growth of the nerve fibers and completely inhibited the formation of end-bulb neuromata. A detailed report of these observations will be the subject of this paper, because it is felt that if a proved successful method could be devised for the prevention of neuroma formation, a considerable clarification of the problem of the painful stump and phantom limb might be effected by the elimination of the neuroma as a causative factor.

Experimental. Methods and Results. The following fundamental procedure was carried out on mongrel dogs. The operations were performed under ether anesthesia and strict asepsis. The skin overlying the intermuscular groove on the lateral aspect of the thigh was incised. The sciatic nerve was exposed, dissected from its areolar tissue bed, and divided. The proximal stump was treated as indicated under the different subdivisions of this experiment. Thereafter, the skin was closed, using a continuous subcuticular suture of fine cotton. Whenever a wound infection occurred, the animal was dropped from the series. After varying periods, these animals were sacrificed and specimens of the nerve were taken for microscopic study. The specimens were fixed in Bodian's[16] No. 2 fixative and the sections were subsequently stained with Bodian's Activated Protargol Stain.[17]

Group I. Control Experiments. This group was comprised of 9 animals. The fundamental operation outlined above was performed, and a segment of the nerve 2 cm in length was excised. The proximal nerve stump was replaced in its bed without further treatment. These animals were sacrificed after a period varying from 30 to 90 days. In all instances typical neuroma formation had occurred (Table I and Fig. 1).

Group II. After performing the basic procedure, the proximal nerve stump was injected with absolute alcohol. This group was comprised of 2 animals; and in both instances, large end-bulb neuromata had formed at the end of 30 days.

Group III. Five animals comprised this group. After performing the basic operation, the proximal nerve stump was placed in a cellophane tube, the distal end of which was closed by a silk ligature. The results in this group are not entirely consistent. In all instances, there was considerable connective tissue reaction, and it was not always possible to identify the cellophane at the end of the experiment. In one instance the nerve, while still within the cellophane tube, showed an atypical diffuse enlargement. Some of the

[9] Corner, E. M., *Proc. Roy. Soc. Med.*, 1918, **11**, 7.

[10] Chapple, W. A., *Brit. Med. J.*, 1918, **1**, 399.

[11] Stookey, B., *Surgical and Mechanical Treatment of Peripheral Nerves*, W. B. Saunders Co., Chap. 21, p. 461, 1922.

[12] Kirk, N. T., *Amputations, Prac. of Surg.*, Dean Lewis, III, Chapter 10, 1943.

[13] Bate, J. T., *Am. J. Surg.*, 1944, **64**, 373.

[14] Herrmann, L. G., and Gibbs, E. W., *Am. J. Surg.*, 1945, **67**, 168.

[15] Poth, E. J., and Fernandez, Bravo E., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 7.

[16] Bodian, D., *Anat. Rec.*, 1937, **69**, 153.

[17] Bodian, D., *Anat. Rec.*, 1936, **65**, 89.

TABLE I.
Experiments Grouped According to Treatment of Sciatic Nerve.

Group	Animal No.	Duration of experiment, days	Result
I. Control—2 cm of sciatic nerve resected	5	90	Large neuroma
	6	90	" "
	7	90	" "
	8	70	" "
	9	30	Small "
	10	45	Large "
II. Nerve stump injected with absolute alcohol	NA 53	15	Small "
	NA 54	30	Large "
III. End of nerve encased in cellophane	NA 12	120	Mass of scar tissue (Neuroma?)
	NA 13	20	No neuroma
	NA 17	200	Enlargement of nerve stump—no neuroma
	NA 20	120	Tapering nerve stump—no neuroma
	NA 23	160	Mass of scar tissue (Neuroma?)
IV. End of nerve placed in silver tube	NA 11	200	Out of tube. Large neuroma
	NA 16	200	Large neuroma
	NA 18	200	" "
	NA 19	200	No neuroma. Considerable scarring around tube
	NA 38	100	Out of tube. Large neuroma
V. End of nerve placed into arterial sleeve	NA 56	75	No neuroma
	NA 57	80	" "
VI. End of nerve placed into snugly fitting glass tube 2½ cm long	1	102	" "
	2	68	" "
	3	102	" "
	4	102	" "
	11	30	Enlarged—no neuroma
	12	65	Nerve out of tube. Moderate sized neuroma
	NA 1	230	No neuroma
	NA 2	230	" "
	NA 3	230	" "
	NA 4	230	" "
	NA 5	230	" "
	NA 7	120	" "
	NA 8	140	Nerve enlarged—out of tube
	NA 9	100	No neuroma
	NA 10	100	" "
VII. Intact sciatic nerve inj. with 10% tannic acid	I-1	8	Degeneration of nerve with fibrosis
	I-3	171	
VIII. Intact sciatic nerve inj. with 2% gentian violet	I-2	8	" " " " "
	I-4	171	
IX. Proximal end of nerve inj. with 10% tannic acid	NA 41	140	No neuroma
	NA 42	110	" "
	NA 43	140	" "
	NA 46	130	" "
	NA 47	15	" "
	NA 48	30	" "
	NA 49	130	" "
X. Proximal end of nerve inj. with 2% gentian violet	NA 28	120	" "
	NA 30	180	" "
	NA 31	180	" "
	NA 32	80	" "
	NA 35	180	" "
	NA 60	80	" "

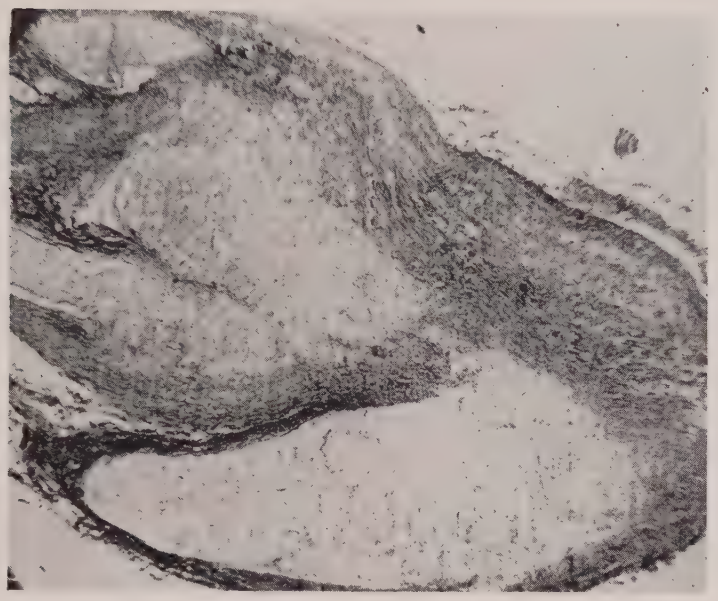

FIG. 1.
The usual neuroma formation 30 days after cutting the sciatic nerve of the dog. Stained with Bodian's Silver Stain. × 60.

fibers had grown beyond the tube and had reanastomosed with the distal segment. These cellophane tubes were thin-walled, non-rigid tubes.

Group IV. Following the basic procedure on the 5 animals comprising this group, the proximal nerve stump was placed within a silver tube, the end of which was closed and folded over on itself. The results in this group were relatively unsatisfactory because the nerves failed to be retained in the tube in all except one instance. Considerable scar tissue reaction occurred around the silver tube. In each instance, where the nerve had pulled out of the tube, a large neuroma had developed.

Group V. Two animals comprised this group. In addition to the basic procedure, the proximal stumps were placed in arterial sleeves. A segment of carotid artery was taken from the same individual animal and washed with heparin. The distal end of the artery was closed by a fine silk tie. Neither of these preparations showed the formation of a neuroma. The remains of the arterial sleeve were visible in the gross specimens.

Group VI. This group was comprised of 15 animals. Following the basic procedure, the proximal nerve stump was placed in a snugly fitting, glass tube about 2½ cm in length. In 2 instances the nerves had pulled out of the glass tubes and showed neuroma formation. In another instance, a large tube was employed, and here again, a neuroma had formed, filling and adapting itself to the confines of the tube. In one instance, where the glass tube had a small, pinpoint opening in the distal end, a fine strand of nerve fibers had grown through this opening and had reanastomosed with the distal segment. In the remaining 11 instances, where the nerves had remained within the snugly fitting glass tube, there was no evidence of neuroma formation. Five of the animals in this group were observed for as long as 230 days.

Group VII and VIII. In the foregoing experiments there is evidence that the formation of end-bulb neuromata can be inhibited by overcoming the growth pressure of nerve axones by fitting the end of a severed peripheral nerve into a rigid tube. The technical accomplishment of this maneuver is difficult and tedious and requires burying foreign substances in relatively large quanti-

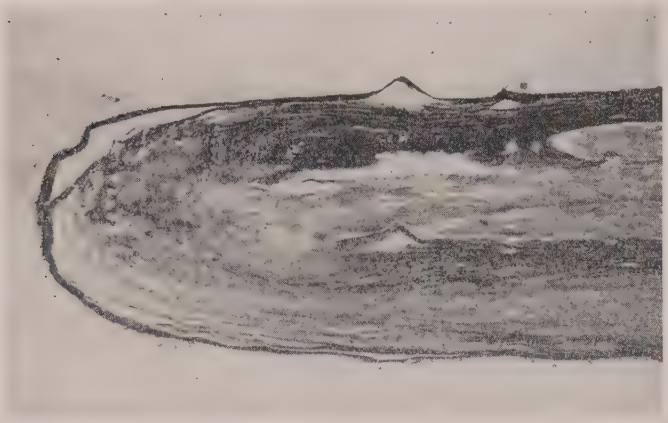

FIG. 2.

Showing the inhibition of the formation of a neuroma when the proximal stump of sciatic nerve had been encased in a snugly fitting glass tube for 230 days. The nerve fibers show little tortuous growth. Stained with Bodian's Silver Stain. × 60.

ties in the tissues. Not only would the procedure be impractical, but the end of the nerve frequently escapes from the encasing tube. Therefore, a search was made for some more practical method. The injection technic was reconsidered. The injection of alcohol does not prevent neuroma formation. The material injected must not cause massive, necrotic destruction of the substance of the nerve, because such a procedure would simulate the simple resection of the nerve at a higher level. The ideal substance for injection should possess some chemical inhibition for the nerve fiber and simultaneously fix or stimulate growth in the fibrous sheaths of the fibers thereby forming enclosing, rigid tubes whose lateral pressure would exert sufficient resistance to overcome the growth pressure of regenerating nerve fibers.

It was found that when tannic acid or gentian violet was injected into intact peripheral nerves, the nerve distal to the site of injection underwent complete degeneration. At the site of injection considerable proliferation of fibrous tissue occurred in what corresponded to the neurilemma. Progressing distally into this area the nerve fibers became more and more isolated and fewer in number until finally they disappeared completely, resulting in total interruption of the nerve pathways.

The sciatic nerve was isolated and a segment about 2 cm in length was injected either with ½% aqueous tannic acid or 2% aqueous gentian violet solution aseptically. These animals were sacrificed at 8- and 171-day intervals and specimens of the sciatic nerve proximal to, at, and distal to the site of injection were fixed in Bodian's No. 2 fixative and stained with Bodian's activated Protargol silver stain.[3]

The results of this study are shown in Fig. 3, 4, and 5.

Group IX. Seven animals comprised this group. Following the basic procedure, the proximal nerve stump was injected with not more than ½ cc of 10% aqueous tannic acid, containing a small amount of methylene blue, which served to stain the injected tissue and indicated the extent to which the nerve was infiltrated. These injections were made at multiple points in an attempt to inject the connective tissue around all fiber bundles. The purpose of this injection was not to destroy the nerve fibers, but rather to fix their surrounding fibrous sheaths to form what would essentially be enveloping, rigid tubes. In no instance could the development of a neuroma be demonstrated. In the case of one animal, however, one intact nerve bundle, which probably escaped injection, became reanastomosed

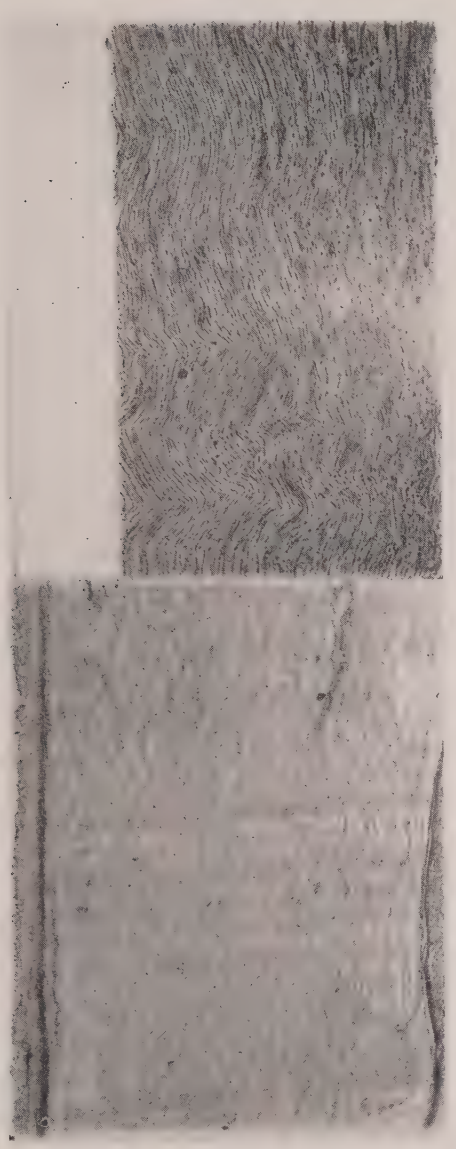

FIG. 3.

Showing the normal fibers of the sciatic nerve of the dog proximal to the site of injection of the nerve with 2% gentian violet 8 days previously. Stained with Bodian's Silver Stain. (*A*, bottom, × 85. *B*, top, × 120.)

with the distal segment.

Group X. Seven animals are included in this group. Following the basic procedure, the proximal nerve stump was injected with not more than ½ cc of aqueous 2% gentian violet, in the same manner as described in Group IX. In no instance was neuroma formation demonstrable. However, again, as in the previous group, a lateral strand of fibers, which had apparently escaped injection, had reanastomosed with the peripheral nerve segment.

A review of the results obtained in these studies indicates that when the end of a severed nerve is supported by a rigid tube, neuroma formation does not occur. Study of microscopic preparations of these nerves injected with gentian violet or tannic acid demonstrates that these substances do not kill all of the nerve fibers. Rather they induce both a chemical inhibition to growth and support the nerve end in a rigid, fixed, connective tissue sheath, which serves the same function as any other snugly fitting fixed support.

The Use of Gentian Violet for Injection of Peripheral Nerve Stump Following Amputation. After demonstrating the efficacy of gentian violet in the inhibition of neuroma formation, all of the severed nerves which could be identified were injected in 15 patients during amputation of the lower extremity. After freeing 2- or 3-inch lengths of the larger nerves, a tight rubber band or a small

FIG. 4.

Same experiment as shown in Fig. 3 and 5. Section at site of injection of 2% gentian violet into the intact sciatic nerve of the dog 8 days previously. Observe the early degeneration of the nerve fibers. Bodian's Silver Stain. × 120.

FIG. 5.

Same experiment as shown in Fig. 3 and 4. This section is taken distal to the site of injection of the intact sciatic nerve of the dog with 2% gentian violet 8 days previously. Note the coiled nerve fragments indicating the degeneration of the axones. Bodian's Silver Stain. × 120. Artifacts present are due to air bubbles in the balsam.

clamp was applied to partially compress the nerve to isolate the freed segment; and gauze was placed around the region during injection to protect the surrounding tissues from the dye, which was inadvertently spilled. The end of the nerve was ligated with fine silk so as not to cut through the sheath but still sufficiently firmly to interrupt the axones. Multiple injections were made into the isolated segment of the nerve to fully distend the sheath. The compressing clamp or rubber band was removed. In the larger nerves from 3 to 5 cc of 2% gentian violet was used. It is desirable always to use a fine caliber needle for the injection. While the number of cases is too small and the lapse of time too short to draw any conclusions as regards the formation of painful stump or tender neuroma, it can be reported that no patient suffered untoward pain because of the procedure, nor has healing been compromised in any way.

Discussion. The proximal end of a severed fiber of a peripheral nerve has the capacity for unlimited growth and will continue to grow until it reunites with an end organ or until its growth pressure is overcome by some other mechanism. The studies by Forssman[18] and Cajal[3] would indicate that a simple mechanical obstacle placed in their path is not sufficient to inhibit growth. Spurling[19,20] effected an orderly, parallel growth of nerve fibers by the use of tantalum foil to surround the site of anastomosis of a peripheral nerve. Weiss[21,22,23] studied the mechanism of nerve regeneration within arterial sleeves. Nerve fibers will grow into a rigid blind tube and completely fill the available space whereupon the fibers cease to grow without undergoing atrophy or degeneration. Likewise, the usual end-bulb neuroma finally ceases to grow when the lateral pressure of the surrounding scar tissue balances the growth pressure of the proliferating nerve fibers. It has been demonstrated again that the injection of ethyl alcohol into the stump of a severed peripheral nerve does not inhibit the formation of end-bulb neuromata.

The procedure of injecting gentian violet into the isolated segment of a severed nerve which has been ligated distally prevents the formation of end-bulb neuromata by both chemical inhibition and mechanical blocking of the pathway of the axones. It having been demonstrated that simple ligation of the central segment is not sufficient to prevent the proliferation of the axones, the additional precaution of injecting the stump with gentian violet is added to give the superimposed effect of chemical inhibitions of nerve regeneration.

Summary. Opposing the growth of nerve fibers by encasing the proximal stump of a severed peripheral nerve in a rigid tube will prevent the formation of an end-bulb neuroma. The injection of the nerve with tannic acid or gentian violet likewise inhibits neuroma

[18] Forssman, J. (Cited by Cajal [3]).

[19] Spurling, R. G., *Surg. Clin. North America*, 1943, **23**, 1491.

[20] Spurling, R. G., *Neurosurg.*, 1944, **1**, 133.

[21] Weiss, P., *Growth* (Supp.), 1941, **5**, 163.

[22] Weiss, P., and Taylor, A. C., *Arch. Surg.*, 1943, **47**, 419.

[23] Weiss, P., and Taylor, A. C., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 77.

formation. The mechanisms probably are not identical. In addition to exerting lateral pressure and limiting the blood supply, tannic acid and gentian violet exert chemical inhibition to the growth of the nerve for a varying period following the injection which further favors the encasement of the resting nerve fibers by a connective tissue envelope.

This method of preventing the formation of end-bulb neuromata may aid in solving the problem of painful amputation stumps and the phenomenon of the phantom limb. No contraindications to injecting the nerve ends in amputation stumps have been observed.

15136

Vaginal Response to Adrenal Stimulation.*

JOSEPH SCHILLER.† (Introduced by F. H. Pratt.)

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The participation of the adrenal cortex in the estrous cycle, established by Wyman,[1] has been confirmed since under various experimental and natural conditions. However, the results of those experiments do not necessarily imply that the adrenal cortex influences the estrous cycle through the release of sex hormones rather than through hormones acting on the general metabolism. The isolation of both estrone (Beall[2]) and of progesterone (Beall and Reichstein[3]) from the adrenal cortex points to the former possibility as a primary factor. In spite of the chemical findings the physiological evidence is scant, and limited to the experiments of Moon,[4] Corey and Britton,[5] Nelson,[6] and Bourne and Zuckerman.[7] The availability of adrenocorticotrophic hormone furnishes an additional technic for this type of investigation.

Material and Methods. Young female rats of the Long-Evans strain were injected daily for 5 days (unless otherwise specified) with adrenocorticotrophic hormone (ACTH).‡ Hepatectomy was performed by making an incision parallel to the ribs and extirpating the median and lateral left lobes. Mortality was 5%. Grafting of the adrenals was done by suturing both glands to the spleen and the "takes" were practically 100%. All sections were stained with hematoxylin-eosin and vaginal smears were taken by the lavage method.

Results. The administration of ACTH to normal females gave inconclusive results. The estrous phase seemed in some instances to be prolonged beyond normal; but since prolonged estrus may occur spontaneously, and since the diestrous phase was unaffected by ACTH, it was concluded that ACTH was ineffective in normal female rats. (Table I.)

On the assumption that the liver might inactivate part or all of the estrogenic substances released by the adrenal cortex

* Aided with a grant by the American Academy of Arts and Sciences from the Permanent Science Fund.

† Present address: Carnegie Institution, Department of Embryology, Baltimore, Md.

1 Wyman, L. C., *Am. J. Physiol.*, 1928, **85**, 414.

2 Beall, D., *J. Endocrinol.*, 1940, **2**, 81.

3 Beall, D., and Reichstein, T., *Nature*, 1938, **142**, 479.

4 Moon, H. D., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 36.

5 Corey, E. L., and Britton, S. W., *Am. J. Physiol.*, 1931, **99**, 33.

6 Nelson, W. O., *Anat. Rec.*, Suppl., 1941, **81**, 97.

7 Bourne, G., and Zuckerman, S., *J. Endocrinol.*, 1941, **2**, 268.

‡ The ACTH was obtained through the courtesy of Dr. E. B. Astwood, and was prepared according to the technic described by R. Tyslowitz (*Science*, 1943, **98**, 225). In all probability the product contained some lactogenic hormone. However, ACTH prepared by us from hog pituitaries according to the technic of G. Sayers, A. White, and C. N. H. Long (*J. Biol. Chem.*, 1943, **149**, 425), which behaves like a pure protein, gave identical results in our hands.

TABLE I.
Vaginal Responses in Young Female Rats Injected Twice Daily with 2 mg of Adrenocorticotrophic Hormone.

No. of group	Type of animal	No. of rats	Vaginal reaction	
			Before ACTH	After ACTH
1	Normal	5	Normal cycle	Unaffected
2	Ovariectomized	5	Leucocytes	"
3	Ovariectomized-adrenalectomized	5	"	"
4	Partially hepatectomized	15	Constant subestrus with epithelial cells predominant	Full cornification
5	Ovariectomized—partially hepatectomized	12	Leucocytes. Rare occasional cornified	Presence of nucleated and mucous cells

(Schiller[8]), further experiments were performed on partially hepatectomized rats. After this procedure, 12 out of 15 animals (Group 4, Table I) went into the condition known as "constant subestrus" characterized by the predominance of epithelial cells in the vaginal smear. Three remained in diestrus throughout the experiment and Loeb's placental sign was negative in all 3. The injection of ACTH in the responsive ones induced full cornification, indicating that the stimulated adrenals can supplement the estrogenic substances sufficiently to induce full estrus.

In order to eliminate the possibility that the injected ACTH had stimulated the pituitary gland to release gonadotropins, five partially hepatectomized rats in constant subestrus were ovariectomized and injected without delay with 4 mg of ACTH daily for 10 days. The vaginal smear continued to show epithelial and cornified cells throughout the experiment. According to Hemmingsen,[9] the estrous cycle once initiated will continue despite ovariectomy. We found this to be true for adrenalectomized and adrenalectomized-ovariectomized rats as well. Groups of 5 normal animals were used and operations were performed during proestrus. However, in no case was a positive smear detectable on the third day after operation, whereas the animals treated with ACTH remained in estrus for 10 days.

[8] Schiller, J., *Endocrinol.*, 1945, **36**, 7.

[9] Hemmingsen, A. M., *Skand. Arch. Physiol.*, 1933, **65**, 97.

Supporting evidence was obtained from Group 5 (See Table I). Twelve partially hepatectomized rats were ovariectomized, permitted to reach anestrus and only then injected with ACTH. Eight only responded to the stimulation. The responsive ones showed in the vaginal smear a predominance of round, nucleated, and mucous cells, and serial sections of the vagina showed areas of stratification (Fig. 1, A) and mucification (Fig. 1, B). The stratification was unevenly distributed along the vaginal wall and full cornification never occurred. Apparently the amount of estrogenic substances released by the adrenal cortex is quantitively insufficient to produce full cornification, but the evidence indicated the presence of subthreshold amounts.

In order to test the hypothesis that the inconclusive results obtained in normal animals were due to the inactivation of adrenal estrogen by the liver, 10 non-hepatectomized female rats were subjected to autoplastic grafting of the adrenals into the spleen. If the liver does inactivate estrogens, then the vaginal smear ought to reflect the same condition as that of adrenalectomized animals. After 6 weeks of salt-water solution for drinking, tap water was substituted and the vaginal smear followed daily. Contrary to expectations 9 animals went into "constant estrus," the cornified cells being predominant. The 10th animal showed regular cycles and even became pregnant, but at autopsy one regenerated cortical mass was found near the left kidney. The spleen-grafted animals subjected to the "resistance to cold" test did not behave differently

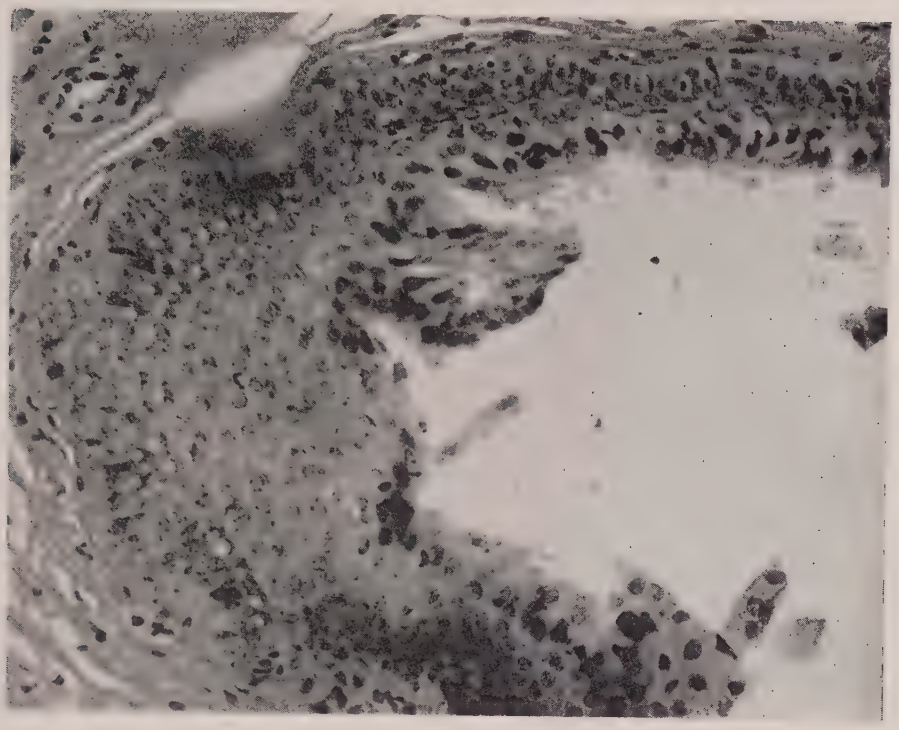

FIG. 1, A.

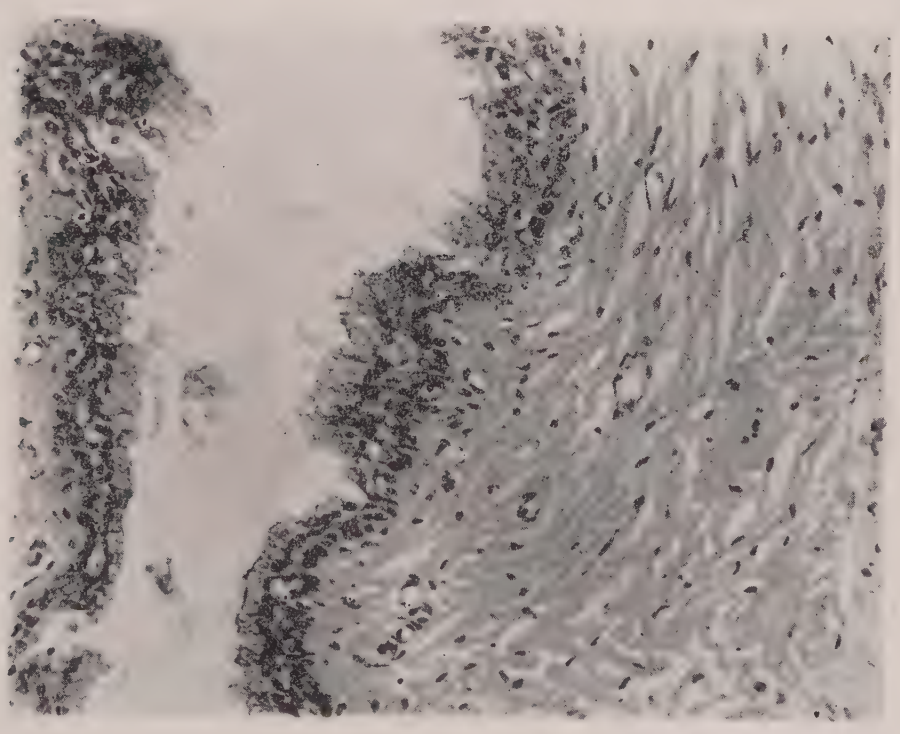

FIG. 1, B.

Stratification (A) or mucification (B) of the vaginal wall after injection of adrenocorticotropic hormone, in ovariectomized rats with subtotal hepatectomy. × 300.

from normal controls. They have been maintained alive without treatment for as long as 3 to 5 months preceding autopsy.

Discussion. The experiments raise several problems. The estrous cycle of normal female rats was not influenced by the administration of ACTH within the dosage used. This contrasts with the results obtained in immature rats. Moon[4] under similar circumstances observed an earlier opening of the vagina, and Nelson[6] an enlargement of the sex accessories. Although the opening of the vagina could be explained in terms of a lower threshold to estrogen stimulation, such would not be the case in Nelson's experiment. It is more plausible to assume either that the metabolism of estrogen assumes different patterns in immature and mature animals, or that the hormonal equilibrium in the normal adult rat cannot be disturbed by ACTH injection. At first sight, the fact that partial hepatectomy facilitates vaginal reactions in young mature rats injected with ACTH would indicate that the amount of estrogenic substances released by the adrenals is quantitively insufficient to overcome the inactivating power of the liver. However, this is not supported by the fact that estrous manifestations are present in non-hepatectomized rats with spleen-grafted adrenals. It is more probable that the metabolism of estrogen follows different patterns in normal and partially hepatectomized rats with regenerating livers.

The stratification and mucification of the vaginal wall in responsive rats indicates that the amount of estrogenic substances released by the stimulated adrenal cortex is too small to induce full cornification. This is in agreement with the findings of Bourne and Zuckerman,[7] who induced cyclic estrus in ovariectomized rats injected with subthreshold doses of estrone, the difference being supplemented by the adrenal gland. Likewise Meyer and Allen[10] obtained mucification of the vaginal epithelium in rodents by using subthreshold doses of estrone. Stratification and mucification of the vaginal epithelium was obtained by Jones and Astwood[11] by using proper amounts of both estrone and progesterone. Since Beall and Reichstein[3] and Beall[2] have isolated these two hormones from the adrenal cortex it is not impossible that our animals were affected by both of them, and by androgens as well. As long as the substances which act on the vagina are not identified, it is impossible to decide which hormone or group of adrenal hormones is responsible for the induced vaginal reaction. If this hormone is a true estrogen, then it differs qualitatively from ovarian estrogen (Golden and Severinghaus[12]) and from exogenous estrogen, which are inactivated by the liver. Parallel conclusions were reached by Burrill and Greene[13] for adrenal androgens and by Eversole, Edelman, and Gaunt[14] for "life maintenance" and "resistance to water intoxication" hormones.

Summary. In partially hepatectomized young female rats the administration of ACTH induces the release of small amounts of hormone from the adrenal cortex, affecting the sex organs.

10 Meyer, R. K., and Allen, W. A., *Anat. Rec.*, 1933, **56**, 321.

11 Jones, G. E. Seegar, and Astwood, E. B., *Endocrinol.*, 1942, **30**, 295.

12 Golden, J. B., and Severinghaus, E. L., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 361.

13 Burrill, M. W., and Greene, R., *Endocrinol.*, 1941, **28**, 874.

14 Eversole, W. J., Edelman, A., and Gaunt, R., *Anat. Rec.*, 1940, **76**, 271.

The technical assistance of Miss T. Landes is acknowledged with appreciation.

15137

Effect of Thiouracil on Growing Swine.*

M. E. Muhrer and A. G. Hogan.

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Hypothyroidism, a condition associated with a decrease in the metabolic rate and with obesity, would conceivably be desirable in animals that were being fitted for market. Andrews and Bullard[1] reported rapid fattening and gain in weight following partial thyroidectomy of steers. In this case the rapid gains may have been partly due to compensation for the loss of weight immediately following the operation as well as to the decreased metabolic rate. The goitrogenic effect of a large number of substances related to thiourea has been demonstrated by Richter and Clisby[2] and also by Kennedy.[3] Astwood[4] found that thiouracil is also very effective in lowering the metabolic rate. These earlier workers were not concerned with economy of gains in their experimental animals but Kempster and Turner[5] reported that the feeding of thiouracil to broiler chicks for 36 days reduced the rate of growth and increased the amount of feed required to produce a pound of gain. The purpose of this investigation was to study the effect of thiouracil upon the economy of gain in swine.

Experimental. The animals were Chester White and Poland China crossbred pigs, paired according to weight, sex, age, and type. Whenever the pairs were of unequal weight the heavier pigs were placed in the control pen. The control ration was made up of: 60 parts corn, 13 parts wheat, 10 parts tankage, 10 parts soybean oil meal, 5 parts alfalfa meal, and 2 parts mineral, by weight. The test ration was the same except for the addition of 0.2% of thiouracil. The animals were hand-fed twice daily and the control and test animals were given the same amount of feed per pig per day. This was less than the control group would have voluntarily eaten but was approximately *ad lib.* feeding for the test animals. The amount of feed consumed and the gains in weight are shown in Table I.

Analysis of variance by the method described by Snedecor[6] shows that the differences were highly significant. The uneconomical gains in Group I (Table I) are partially due to the poor quality of the corn that it was necessary to use at the time. The very rapid and economical gains reported in Group II were due to the fact that these animals had been somewhat underfed during the month before the experimental period started. All of the animals in Group III had previously been on another experiment and they had received some thiourea before being placed in the trials referred to in Table I. The previous management of the animals in Group IV had been in accord with recommended practices.

Discussion. Table I summarizes some of the effects of thiouracil on the utilization of food by swine. When the consumption of food and of energy was the same, the animals which received thiouracil gained more rapidly in weight than did the controls. In other words, they required less food per unit of gain.

The controls had a much better appetite than did the test animals and they would have consumed a much larger quantity of food if it had been available. A higher feed intake

* Aided by a grant from the John and Mary R. Markle Foundation and in cooperation with the Animal Husbandry Department, Missouri Agricultural Experiment Station.

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1 Andrews, F. N., and Bullard, J. F., *Proc. Am. Soc. An. Prod.*, 1940, p. 112.

2 Richter, C. P., and Clisby, K. H., *Arch. Path.*, 1942, **33**, 46.

3 Kennedy, T. H., *Nature*, 1942, **150**, 233.

4 Astwood, E. B., *J. A. M. A.*, 1943, **122**, 78.

5 Kempster, H. L., and Turner, C. W., *Poultry Science*, 1945, **24**, 94.

6 Snedecor, G. W., *Calculation and Interpretation of Analysis of Variance and Covariance*, Collegiate Press, Inc., Ames, Iowa, 1934.

TABLE I.
Effects of Thiouracil upon the Economy of Gain of Swine

Groups	Treatment	No. of animals	Initial wt (lbs)	Wt after 28 days (lbs)	Gain (lbs)	Avg daily gain (lbs)	Feed consumed (lbs)	Lbs feed/ 100 lbs gain
I	Control	3	152	188	36	1.3	231	642
	Treated*	2	151	210	59	2.1	231	392
II	Control	3	149	203	54	1.9	198	367
	Treated*	3	120	196	76	2.7	198	261
III	Control	4	143	178	35	1.3	143	409
	Treated*	4	137	191	54	1.9	143	265
IV	Control	7	71	99	28	1.0	112	400
	Treated*	4	70	120	50	1.8	112	224
Combined I, II, III and IV	Control	17	129	167	38	1.4	183	482
	Treated*	13	120	179	59	2.1	183	310

*.2% of ration was thiouracil, kindly furnished by Lederle Laboratories.

would have increased the rate of gain but it is highly improbable that the rate of gain, or the gain per unit of feed consumed, would ever have been higher than that of the thiouracil-fed pigs. A daily gain of 2.03 lb on an unrestricted diet was observed by Hogan *et al.*[7] which is as high as has ever been reported in the literature that has come to our attention, and is of the same order as the gains made by the thiouracil-fed pigs. However, the pigs on the unrestricted diet required 3.39 lb of feed per pound gained, which is less than was required by our controls, but was more than was required by the pigs which received thiouracil.

Although the animals which received thiouracil gained more than the controls the increased gain was not true growth. The heavier thiouracil-fed pigs were wider, shorter, and not as tall as the controls. They were apparently an example of retarded growth with rapid gains in weight due to deposition of an excessive amount of fat. The low cost of gains by the treated swine is probably due to the lower metabolic rate observed by Astwood.[4] It was also noted that the controls were much more active than the test animals. The treated animals seemed to sleep a greater portion of the time while the controls were active, which would also be a factor in the difference in cost of gain. This is in agreement with the observations of Andrews and Bullard[1] on steers, but the results on the swine are independent of an immediate loss in weight from surgical shock.

Our results are not concordant with those of Kempster and Turner[5] who observed that thiouracil increased the feed intake and decreased the gain in weight of growing chickens. The different effects produced by this drug on different species and under varying conditions within a species make additional investigations in this field desirable. These should include the length of time thiouracil is retained in the tissues and the possibility that if thiouracil-fed animals were slaughtered too soon their flesh would be unsuitable for human food.

Summary. 1. Four groups of swine were pair-fed on rations which were identical except for the addition of 0.2% thiouracil to the ration of the test animals. 2. The test animals were less active, and after 28 days they were shorter and fatter than the controls. They gained weight more rapidly and more economically than did the controls.

[7] Hogan, A. G., Weaver, L. A., Edinger, A. T., and Trowbridge, E. A., *Missouri Experiment Station Research Bulletin* 73.

15138

No Androgen in Corn Pollen.*

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In connection with another investigation, we had the opportunity to apply to corn pollen a procedure found to be efficient for the extraction of androgens from animal material. Although no androgen was found upon assay of the extracts, it seems desirable to record the procedures employed. The main interest in the negative finding is that androgenic substances have not been reported to occur in plant materials, in contrast with the occurrence in plants of substances exhibiting estrogenic activity.

The pollen was collected by hand at Beltsville in August, 1944, chiefly from the hybrid US-13. Immediately after collection it was dried at 40°C, then kept in a covered glass jar at 0°C, and used 4 months later. Just before extraction the pollen was dried to constant weight at room temperature in vacuum over sulfuric acid.[1] The weight loss due to this last operation was 7.4%. Two portions, 139 g and 324 g, were taken. Each of these was worked up differently in order to insure adequate extraction of all components with properties similar to the known animal androgens and their inactive conjugates. The pollen was well ground under the solvents used for extraction.

Extraction. The smaller portion was thoroughly extracted by shaking with water at 40°C. The aqueous extract was fractionated by a procedure which has been found satisfactory for the investigation of urine.[2] This includes heating with barium chloride to liberate any androgen which may be present in inactive conjugated form. The water-insoluble residue, after thorough drying, was extracted with petroleum ether of boiling range 39-43°C. The solid residue, insoluble in petroleum ether, was subjected to the same treatment as the *n*-butanol extractives in the procedure of Talbot *et al.*[2] Hence two solutions in carbon tetrachloride and one in petroleum ether were prepared from the smaller portion of pollen.

The larger portion of pollen was thoroughly extracted in succession with petroleum ether and ethanol. The ethanol-insoluble solid was then fractionated in the same way as the smaller portion of whole pollen. This yielded finally 3 solutions in carbon tetrachloride and 2 in petroleum ether as shown in the accompanying scheme.

The carbon tetrachloride solutions were washed with water and dried with calcium chloride. The solvents were evaporated from the 8 solutions, and as much of the solvent-free extractives as possible (20 to 21 g) was dissolved in corn oil (Mazola), the last parts by heating on a steam-bath (80-90°C) for 30 minutes, to make 80 ml. This solution after cooling remained clear for several months; after 4 months the sediment which had deposited amounted to less than a gram.

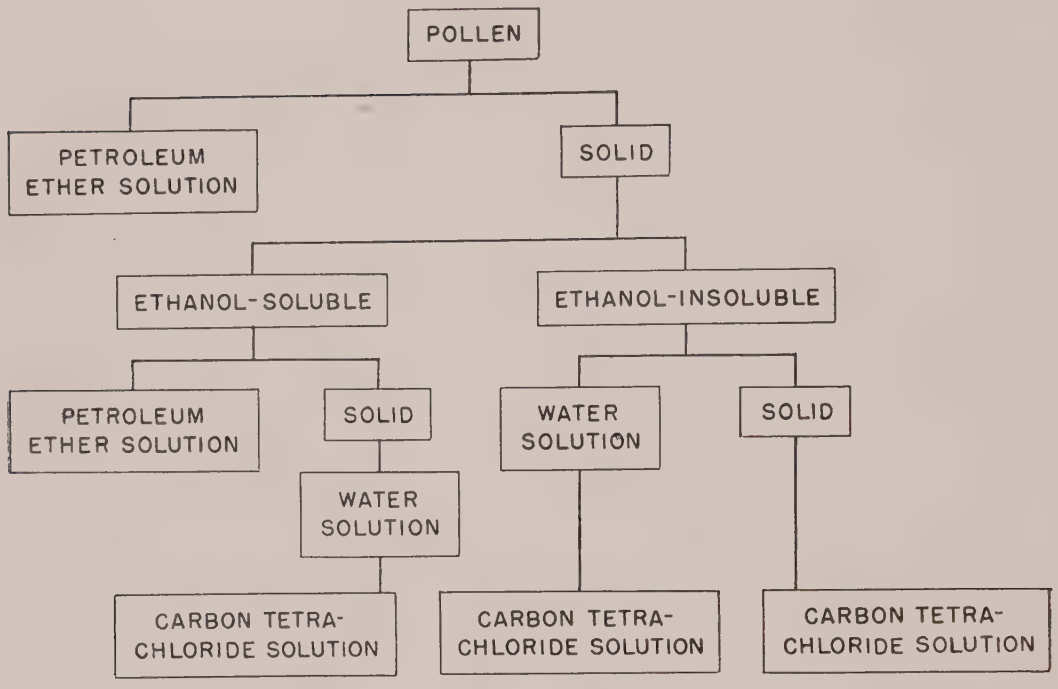
Although any androgens similar to the known chemical types would be found in the corn oil solution, an attempt was made to assay also the extractives which did not dissolve since the quantity was large (not accurately determined, but about 20 g). This material was dissolved in ethanol to make 160 ml, but changes occurred in this solution, for after 3 days most of the material had precipitated out. The remaining solution, which contained 1.45 g of material not volatilized at 90°C and a slightly reduced pressure (this was determined on a small aliquot),

* This work was supported in part by an allotment from the Special Research Fund (Bankhead-Jones Act of June 29, 1935).

† We are glad to acknowledge the collaboration of Mr. Adam M. Gaddis, who made the extracts, and that of Mr. Benjamin H. Neher, who carried out the tests on capons.

1 Anderson and Kulp, *J. Biol. Chem.*, 1922, **50**, 434.

2 Talbot, Ryan, and Wolfe, *J. Biol. Chem.*, 1943, **148**, 596.



was assayed both without further treatment and after removal of most of the ethanol.

Any water-soluble androgen not appearing in either the carbon tetrachloride or petroleum ether solutions obtained by the procedure described would have been missed in this work. Such androgens are unknown and therefore no methods for their bioassay have been developed.

Assays. The comb response of the Brown Leghorn capon was used for testing the extracts for androgenic activity. Both the corn oil and ethanol solutions were applied directly to the comb and injected subcutaneously.

Direct application to the comb was made with a syringe and needle, the material being spread as uniformly as possible over the entire surface of the comb. One to 3 applications were made daily, the number of applications depending upon the quantity administered and the consistency of the preparation.

The subcutaneously administered preparations were injected beneath the skin of breast areas, one-half the total quantity to each side. The material in ethanol was injected in the form of a reasonably homogeneous emulsion made by shaking the solution with 4 volumes of water just before injection.

TABLE I.

Effect of Extracts of Corn Pollen on the Comb of the Brown Leghorn Capon.

Test No.	Extract	Administration: Quantity	Administration: Route	Capons No.	Change in comb dimensions* mm
1	Ethanol sol.	0.2 ml	Direct	4	+0.50
2	" "	0.6 "	"	4	—2.50
3	" "	2.0† "	Subcutaneous	5	+0.25
4	" " conc.	0.2‡ "	Direct	3	—1.01
5	Corn oil sol.	0.6 "	"	4	—1.25
6	" " "	1.0 "	Subcutaneous	4	—0.25
—	Androsterone	0.2 mg	Intramuscular	9	+10.12

* Total change in length plus height found on days 6-8 for the tested preparations, day 6 for androsterone.

† 2.0 ml of the ethanol solution was mixed with 8.0 ml water daily just before injection.

‡ 0.2 ml of the concentrate is the equivalent of *ca.* 3.0 ml of the ethanol solution.

Control injections of androsterone were made intramuscularly into the breast in accordance with the method of Parkes and Emmens.[3]

Extracts under test were administered over 6 days, the androsterone over 5 days. Change in comb size was calculated as the sum of the changes in length and height occurring between the first day of treatment and the first or second day following the last administration.

The results of the assays are given in Table I. No androgenic response was observed after administration of either of the pollen extracts. The response of the capons to androsterone was almost exactly that previously observed by others.[3] It can be calculated from the figures presented that the smaller doses administered would have given a positive response if the corn pollen had contained 0.003% or more of an androgen as potent as androsterone and if 30% of such an androgen had been extracted by the procedure employed.

Summary. Extraction of corn pollen and assay of the extracts according to procedures commonly used on animal materials failed to yield any evidence of androgenic substances.

3 Parkes and Emmens, *Vitamins and Hormones*, Harris and Thimann, Editors, Academic Press, Inc., New York, 1944, **2**, 376.

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Incidence of the Eight RH Types Among 179 White Puerto Ricans.

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In the early work on the Rh factor, it was noticed that some bloods might be classified as Rh positive or Rh negative depending upon the human anti-Rh serum employed. It has been shown that human anti-Rh sera vary in their specificity, because they contain antibodies resulting from immunization to different Rh antigens. The anti-Rh sera that have thus far been found contain the following antibodies: anti-Rh_0, anti-Rh′, anti-Rh″, anti-Rh'_0, and anti-Rh''_0. The anti-Rh_0 sera correspond in specificity with anti-Rh sera obtained by immunizing animals with *Macacus rhesus* blood. The anti-Rh'_0 contain two antibodies, anti-Rh_0 and anti-Rh′; this holds true for anti-Rh''_0 containing antibody anti-Rh_0 and anti-Rh″.[1] Anti-Rh sera containing other antibody combinations have been found by British authors.[2]

Employing the 3 different varieties of human anti-Rh sera containing only one antibody, 8 Rh types have been defined by Wiener,[3] Rh_0, Rh_1, Rh_2, Rh_1Rh_2, Rh′, Rh″, Rh′Rh″, and Rh negative. Type Rh_1 may also be designated as Rh'_0, Type Rh_0 as Rh''_0, Type Rh_1Rh_2 as $Rh'_0Rh''_0$ to indicate the antibodies with which they react. (See Table I.)

There are some bloods giving weak reactions with some of these three varieties of anti-Rh sera; they have been classified as intermediate Rh types $Rh_1('')$, $Rh_2(')$, $Rh_0('')$, and $Rh_0(')$ (Wiener[4]). (Table II.) Murray, Race, and Taylor[5] have also described a rare agglutinogen, Rh_z.

The distribution of the various Rh blood types among white individuals in New York City and among Negroes can be seen in Table III.

With the data here presented, a study has been undertaken to determine the incidence

1 Wiener, A. S., *Am. J. Clin. Path.*, 1945, **15**, 106.

2 Stratton, T., *Nature*, 1944, **153**, 773.

3 Wiener, A. S., *Science*, 1944, **99**, 532.

4 Wiener, A. S., *Science*, 1944, **100**, 595.

5 Murray, J., Race, R. R., and Taylor, G. L., *Nature*, 1945, **155**, 112.

FIG. 1.

TABLE I.

The Eight Standard Rh Types (After Wiener).

Rh Anti-sera			
Anti-Rh_0 Negative	Anti-Rh′ Negative	Anti-Rh″ Negative	Rh Type Negative
+	+	Neg.	Rh_1
+	Neg.	+	Rh_2
+	Neg.	Neg.	Rh_0
+	+	+	Rh_1Rh_2
Neg.	+	Neg.	Rh′
Neg.	Neg.	+	Rh″
Neg.	+	+	Rh′Rh″

TABLE II.

Intermediate Rh Types (After Wiener).

Intermediate types	Reactions with anti-sera			Formerly classed together with major types
	Anti-Rh_0	Anti-Rh′	Anti-Rh″	
$Rh_1(″)$	Positive	Positive	Weak	Rh_1
$Rh_2(′)$	Pos.	Weak	Pos.	Rh_2
$Rh_0(″)$	Pos.	Neg.	Weak	Rh_0
$Rh′_0$	Weak	Pos.	Neg.	Rh′

TABLE III.

Distribution of the Rh Types Among Whites and Negroes in New York City* and White Puerto Ricans.

			Rh Types.							
No. tested	Race	Location	Rh negative	Rh_0	Rh_1	Rh_2	Rh_1Rh_2	Rh′	Rh″	Rh′Rh″
1000	White	N. Y. City	12.9	2.6	54.1	12.8	16.4	0.9	0.3	—
223	Negro	”	8.1	41.2	20.2	22.4	5.4	2.7	—	—
179	White	Puerto Rico	10.06	15.08	39.10	19.54	13.97	1.68	.55	—

* From Wiener, A. S., Davidson, L., and Potter, E. L., *J. Exp. Med.*, 1945, **81**, 63.

of the Rh types among the white Puerto Rican population. Table III also shows the results of a study to determine the Rh types of 179 white individuals; the bloods have been obtained from blood donors and hospital employees coming from different parts of the island.

Rh type determinations have been performed employing anti-Rh_0, anti-Rh′, and anti-Rh″ sera;* 100 of these determinations were performed with anti-$Rh′_0$ and anti-$Rh″_0$ sera; to prepare the anti-Rh′ and anti-Rh″ sera blocking anti-Rh_0 serum was added in a proportion suggested to us by Dr. Wiener[6] with the purpose of eliminating the action of the anti-Rh_0 antibody. The rest of the Rh types were determined by employing pure anti-Rh′ and anti-Rh″ sera so that it was not necessary to add blocking serum because anti-Rh_0 agglutinins were absent.

It can be observed that the percentage of the different Rh types in Puerto Rico varies from those obtained in the United States. We have found about 15% Rh_0 individuals as compared to 2.4% among white Americans; type Rh_2 also has a higher incidence, while type Rh_1 represents only 39.1% compared to 54.4% obtained by Wiener in New York City. In Puerto Rico there are only about 10% Rh negative individuals. Twenty-two of the 179 bloods studied gave weakly positive reactions with one of the anti-Rh sera used

* Obtained through the courtesy of Dr. Alexander S. Wiener, of the Office of the Chief Medical Examiner of New York City.

6 Personal communication.

and perhaps should have been classified as intermediate types. Intermediate types are frequent in Negroes.[7]

[7] Wiener, A. S., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 89.

The difference in incidence of the Rh types may well be explained on the basis of some unknown mixture with the colored race, which differs in regard to the incidence of the different Rh types from the white American population.

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Influence of Sulfonamides and Para-aminobenzoic Acid on the Growth of *Lupinus albus.*

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The mode of action of sulfanilamide is still an unsolved problem. Various hypotheses based more or less on experimental data have been advanced, as for instance the catalase hypothesis, the respiration-interference hypothesis, the oxidation hypothesis, and others, described by Long[1] and by Henry.[2] Perhaps the most widely accepted explanation is the so-called PABA theory. Since under certain conditions para-aminobenzoic acid (PABA) neutralizes the bacteriostatic effects of sulfanilamide, it has been suggested that the influence of PABA is a result of its competition with sulfanilamide for a place in certain enzyme systems of susceptible bacteria. In the use of seedlings and other botanical test objects for pharmacological studies[3,4] it seemed of interest to investigate the effects of para-aminobenzoic acid on the one hand and of sulfonamides on the other, separately and in combination with each other, on the growth of higher plants. The results have been partially reported[5] and are given more fully here.

Method. The biological criterion employed was the elongation or growth of the well-defined straight roots of Lupinus seedlings, studied by the author's phytopharmacological methods, details of which have been published.[6] The procedure was briefly as follows.

Seeds of *Lupinus albus,* large variety, were soaked in tap water overnight and planted in finely ground sphagnum moss containing adequate moisture. After germination, the growth of selected seedlings with roots 35 to 50 mm long was studied in a solution consisting of equal parts of distilled water and Shive physiological saline.[7] The various drugs, sulfonamides and PABA, were dissolved in the same hydroponic medium, *i.e.,* half distilled water and half Shive solution. At least 10 seedlings, placed in hard-glass test tubes, were employed for the control solution and for each drug solution, in the different concentrations used. The sharply defined straight roots were measured at the beginning and end of every experiment, and the ratio of growth of the seedling roots in the various drug solutions is expressed as a percentage of their growth in the control solution according to the formula,

$$\text{Index of Growth} = \frac{X}{N} \times 100$$

in which N represents average growth of controls and X the average growth in the drug solution. All of the seedlings were allowed to grow for 24 hours in the dark at 15°C.

Substances Studied. The investigation may

[1] Long, P. H., *Sigma Xi Quarterly,* 1941, **29**, 149.

[2] Henry, R. J., *Bact. Rev.,* 1943, **7**, 175.

[3] Macht, D. I., *J. Pharm. and Exp. Ther.,* 1926, **29**, 461.

[4] Macht, D. I., *Science,* 1930, **71**, 302.

[5] Macht, D. I., and Kehoe, D. B., *Fed. Proc.,* 1943, **2**, 30.

[6] Macht, D. I., and Macht, M. B., *J. Lab. and Clin. Med.,* 1941, **26**, 597.

[7] Shive, J. W., *Physiol. Rev.,* 1915, **1**, 327.

be divided into three parts: (1) The influence of benzoic acid and of its ortho-, meta-, and para-amino derivatives; (2) the effect of a number of sulfonamides in various concentrations, including sulfanilamide, sulfathiazole, sulfapyridine, sulfadiazine, sulfaguanidine, succinyl sulfathiazole, and neoprontosil; (3) a more extensive study of sulfanilamide in various concentrations, of PABA in various concentrations, and of mixtures of the two, to ascertain their effect on each other.

Results. Effect of Benzoic and Amino-benzoic Acids. Benzoic acid and sodium benzoate have been previously studied by Macht and Livingston, and were found to be exceedingly toxic for plants.[8]

Twenty experiments were performed on the comparative toxicity for the *Lupinus albus* seedlings of the 3 amino-benzoic acids. The pH of the various concentrations employed in Shive's solution varied but little (pH 4.8-pH 5.2) and it has been previously determined that even much wider differences in the pH of Shive solutions had little effect on the growth of lupines. Of the 3 amino-benzoic acids the meta variety was the most toxic, and the para variety the least toxic. For illustration, the growth index of Lupinus seedlings in concentrations of 1:20,000 dissolved in Shive solution was 31 for *m*-benzoic acid, 50 for *o*-benzoic acid, and 68 for PABA.

It will be shown later that PABA, while inhibiting growth in the above concentration (1:20,000), actually stimulates growth in highly dilute solutions.

Effects of Various Sulfonamides. Of the 7 sulfa compounds studied for comparative purposes 20 experiments were made with each. Growth in fairly high concentration (1:10,000) revealed that sulfanilamide produced the greatest inhibition (Table I). Sulfathiazole, sulfapyridine, and sulfadiazine were less inhibitory in 1:10,000 while sulfaguanidine, succinyl sulfathiazole, and neoprontosil were actually stimulating to root growth in that concentration.

This stimulating effect of the last 3 compounds prompted further study of the first 4, namely, sulfanilamide, sulfathiazole, sulfapyridine, and sulfadiazine in weaker dilutions. It will be seen (Table I) that those 4 sulfonamides which produced some inhibition of growth in concentration of 1:10,000, also produced stimulation at lower concentrations. This biphasic or diphasic action of drugs on plant growth is not an unusual phenomenon in plant physiology and pharmacology. Thus, the 3 auxins or plant hormones, indole-acetic, indole-butyric, and naphthalene acetic, acids while stimulating root growth in very weak doses, actually inhibit growth when used in greater concentrations.[9] The statistical reliability of the figures obtained with 10 or more plants in any one experiment has been fully discussed elsewhere[6] and the same degree of validity applies to the data of the present research. As regards the comparative data obtained in different experiments performed with different crops of plants on different days the variations in the results did not vary more than 5 to 10%.

TABLE I.
Effect of Higher Dilutions of Aqueous Solutions of Various Sulfa-Compounds on Growth of Lupinus Seedlings.

Drug	Concentration × 10,000	Phytotoxic index
Sulfanilamide	1:1	68
,,	1:10	86
,,	1:25	112
Sulfathiazole	1:1	86
,,	1:10	90
,,	1:25	118
Sulfapyridine	1:1	88
,,	1:10	94
,,	1:25	130
Sulfadiazine	1:1	87
,,	1:10	113
Sulfaguanidine	1:1	114
Succinyl sulfathiazole	1:1	113
Neoprontosil	1:1	137

Effect of Sulfanilamide and PABA in Different Concentrations. Inasmuch as most of the previous work on the mode of action of sulfonamides has been done with sulfanilamide, the effect of sulfanilamide and PABA separately and together in various concentrations was next studied more intensively.

Sixty experiments were performed with each

[8] Macht, D. I., and Livingston, M. B., *J. Gen. Physiol.*, 1922, **4**, 573.

[9] Macht, D. I., and Grumbein, M. L., *Am. J. Botany*, 1937, **24**, 457.

TABLE II.
Growth of *Lupinus albus* Seedlings

In aqueous solutions of sulfanilamide		In aqueous solutions of PABA	
Concentration × 10,000	Index of growth	Concentration × 10,000	Index of growth
1:1	68	1:0.1	19 (plasmolysis)
1:2.5	73	1:1	60
1:5	77	1:2	64
1:10	94	1:2.5	73
1:20	100	1:5	77
1:25	106	1:10	80
1:50	110	1:20	82
		1:25	84
		1:50	88
		1:100	107
		1:125	110

TABLE III.
Growth Indices for Combination Experiments with Lupinus Seedlings.

Experiment	PABA dilution	Sulfonamide dilution	PABA and sulfa (equal parts)
I	25,000 ×	250,000 ×	57%
G.I.	70%	94%	
II	500,000 ×	250,000 ×	74%
G.I.	86%	100%	
III.	500,000 ×	25,000 ×	51%
G.I.	82%	81%	
IV	250,000 ×	10,000 ×	50%
G.I.	84%	71%	
V	25,000 ×	25,000 ×	63%
G.I.	72%	78%	
VI	250,000 ×	250,000 ×	53%
G.I.	78%	100%	
VII	25,000 ×	250,000 ×	72%
G.I.	72%	105%	
VIII	1,000,000 ×	1,000,000 ×	97%
G.I.	105%	107%	

drug and a summary of the results is shown in Table II. It will be seen that sulfanilamide exerts an inhibitory effect on growth in concentrations below 1:100,000 but that in weaker solutions it is mildly stimulating to growth.

With para-aminobenzoic acid, a diphasic action may also be noted; in suitable concentrations it stimulated root growth.

When various concentrations of the 2 drugs were used together 1:1 a definite *synergism* or potentiation was noted in every instance, not only when the drugs were dissolved in Shive solution, but also when they were dissolved in *water* without admixture of plant-physiological electrolytic salts. These data are obviously quite unlike those reported by observers who used mixtures of the same drugs on bacteria. (Table III.)

Comment. The PABA theory for the mode of action of sulfanilamide as advanced by the original workers, Lockwood,[10] Woods,[11] Fildes,[12] Bliss and Long,[13] and others, is probably the most satisfactory explanation so far offered. It is based on the cardinal fact that para-aminobenzoic acid antagonizes the action of sulfanilamide on bacteria and other organisms. Thus, Maier and Riley[14] found it to inhibit the sulfanilamide effect on plasmodia, and similar observations were made

[10] Lockwood, J. S., *J. Immun.*, 1938, **35**, 155.

[11] Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

[12] Fildes, P., *Brit. J. Exp. Path.*, 1941, **22**, 293.

[13] Bliss, Eleanor A., and Long, P. H., *Bull. Johns Hopkins Hosp.*, 1941, **69**, 14.

[14] Maier, J., and Riley, E., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 152.

by Marshall, Litchfield, and White,[15] and by Seeler, Graessale, and Dusenberg.[16] PABA counteracts the inhibiting effect of sulfanilamide on cell division of the flagellate *Polytomella caeca*.[17] Similar antagonism is exerted by para-aminobenzoic acid on fresh water diatoms;[18] on pigment formation of *Pseudomonas seruginosa*;[19] on the effect of sulfanilamide on experimental *Lymphogranuloma venereum* virus,[20] and on some yeasts.[21]

It is therefore surprising that the influence of sulfanilamide and PABA combinations on higher plants such as *Lupinus albus* is diametrically opposite and the converse of antagonism.

This is of interest from the standpoint of general biology. In the earlier days of bacteriology it was generally held that bacteria are microscopic plants. In fact, older writers often resorted to yeast cultures for testing the antiseptic properties of drugs. In the light of our present findings the behavior of bacteria under the combined action of sulfanilamide and PABA resembles more the effects produced by these drugs on plasmodia and flagellates which are commonly classed as animal organisms. The results yielded by a study of *Lupinus albus* emphasize the uncertain demarcation between the lower animal and plant organisms. The present findings illustrate well the dangers of hasty and sweeping generalizations in pharmacology. It is hazardous to apply deductions from data of one species of zoo- or phyto-pharmacological test-objects to another, without experimental tests in support of such conclusions.

Summary. Pharmacological studies on root-growth of *Lupinus albus* seedlings under standardized and rigidly controlled conditions reveal that: (1) Of the 3 amino-benzoic acids the *meta* isomer is the most toxic, and the *para* isomer is the least toxic. (2) Para-aminobenzoic acid exerts a diphasic effect on root-growth: Very weak dilutions are growth-promoting, while stronger concentrations are growth-inhibitory. (3) Sulfanilamide also exhibits a similar action, some concentrations inhibiting growth while other concentrations promote it. (4) Similar findings were made with 6 other sulfonamides which were examined. (5) Combinations of sulfanilamide and para-aminobenzoic acid in every case exerted not an antagonistic, but on the contrary, a synergistic or potentiated depression on growth of *Lupinus albus* roots.

15 Marshall, E. K., Jr., Litchfield, J. T., Jr., and White, H. J., *J. Pharm. and Exp. Ther.*, 1942, **75**, 89.

16 Seeler, A. O., Graessale, O., and Dusenberg, E. D., *J. Bact.*, 1943, **45**, 205.

17 Lwoff, A., Nitti, F., Trefowel, Mme. J., et Hamon, V., *Ann. Inst. Pasteur*, 1941, **67**, 9.

18 Wielding, S., *Science*, 1941, **94**, 389.

19 Ungar, J., *Nature*, 1943, **152**, 22.

20 Findlay, G. M., *Brit. J. Exp. Path.*, 1940, **21**, 356.

21 Landy, M., and Dicken, D. M., *Nature*, 1942, **149**, 244.

15141

Cause of an Outbreak of Encephalitis Established by Means of Complement-Fixation Tests.*

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On 16 July, 1945, members of the staff of Naval Medical Research Unit No. 2 arrived on Okinawa to study an outbreak of encephalitis which had appeared among natives of the island during the first week in July. The danger that the encephalitis might interfere with military and naval operations in the Ryukyus made it imperative that the etiologic agent be identified as soon as possible so that proper control measures could be instituted.

* The Bureau of Medicine and Surgery of the Navy does not necessarily endorse the views set forth in this paper.

Because of the proximity of Okinawa to Japan, and because the disease had appeared in midsummer, it was thought that the active agent most likely to be involved was Japanese B encephalitis virus. It was believed that an attempt to demonstrate the development during convalescence of complement-fixing antibodies against this and other neurotropic viruses offered the best means of securing prompt identification of the virus responsible for the outbreak. Accordingly, acute and convalescent sera from patients ill with encephalitis on Okinawa were sent to the Guam laboratories of Naval Medical Research Unit No. 2 where they were tested by the method described by Casals[1] for the presence of complement-fixing antibody against Japanese B and other neurotropic viruses. On July 23, 2 specimens of serum from each of 3 Okinawan patients reached Guam and were tested the following day. Both specimens from 2 of these patients (No. 1 and No. 3) were negative. Serum of the third patient (No. 2) obtained on the eighth day of disease was negative but serum obtained 14 days after he became ill was positive for complement-fixing antibody against the Japanese B virus in a final dilution of 1:16. This indicated that the patient had developed antibodies against this virus as the result of his illness, and that the disease from which he was convalescing was caused by Japanese B virus. It was, therefore, demonstrated within 24 hours after the first samples of serum reached the laboratory that Japanese B virus was present on Okinawa and was responsible for at least a portion of the encephalitis occurring there. Subsequent complement-fixation tests carried out with sera obtained from 17 additional patients on Okinawa who were believed to be suffering from encephalitis have established beyond doubt that a number of them were recovering from infection with this virus.

Results of complement-fixation tests carried out with sera from patients on Okinawa are summarized in Table I. Examination of this table reveals that 6 patients (No. 2, No. 19, No. 30, No. 31, No. 36, and No. 61) showed no complement-fixing antibody against Japanese B virus early in their illness, but developed a significant titer against this active agent between the 14th and 27th day of disease. A 2-plus or greater reaction in a final serum dilution of 1:8 is significant, and these 6 patients developed a titer of 1:16 or greater. It is certain, therefore, that they were convalescent from Japanese B encephalitis. Results obtained with early and convalescent sera from a second group of 5 patients (No. 6, No. 7, No. 28, No. 33, and T.W.H., an American soldier) showed that these persons had developed a significant increase in titer of antibody against Japanese B virus during the time which elapsed between the collection of the first and second specimens of their sera. It is practically certain that these patients also had suffered infection with Japanese B virus.

It is also shown in Table I that sera obtained from patients No. 15, No. 16, and No. 52 were positive 12 to 14 days after they became ill, but that no increase in complement-fixing antibody was observed during the next 8 to 15 days. The significance of the findings in this group is not clear, and is open to several interpretations which will not be discussed here. Only sera drawn late in convalescence from patients H-4 and No. 41 were available for study. The high titer of antibody in their sera indicates that they probably had had recent experience with Japanese B virus, but the relation of this antibody to their illness cannot be determined with certainty.

An examination of Table I also reveals that all sera of patients No. 1, No. 3, No. 27, and No. 39 were negative, indicating that their illness was not Japanese B encephalitis, or that if they had been infected with this virus they had failed to produce antibody against it. It is of interest in this connection to note that autopsy of patient No. 3 proved that his central nervous system symptoms were due to tuberculous meningitis.

It is known that laboratory animals hyperimmunized by vaccination with Japanese B virus develop a high titer of complement-fixing antibody against this virus and a low titer against the virus of St. Louis encephalitis.[2]

1 Casals, J., and Palacios, R., *J. Exp. Med.*, 1941, **74**, 409.

2 Casals, J., *J. Exp. Med.*, 1944, **79**, 341.

TABLE I.

Summary of Complement-Fixation Tests with Sera of Patients on Okinawa Showing Symptoms of Encephalitis.

Date of test	Case	Day of disease serum obtained	Japanese B virus antigen Final serum dilution 1 to: 4	8	16	32	64	128	Western equine Virus antigen Final serum dilution 1 to: 4	8	16	32	64	128	St. Louis Virus antigen Final serum dilution 1 to: 4	8	16	32	64	128	Serum Control
7/24	2	8	0	0	0	0	0														0
24	2	14	4+	3+	2+	±	0														0
8/ 4	2	8	0	0	0	0	0														0
4	2	21	3+	3+	2+	±	0		0	0	0	0	0		0	0	0	0			0
19	30	4	0	0	0	0															0
19	30	20	4+	4+	3+	±	0	0	0	0	0	0	0								0
19	19	10	0	0	0	0															0
19	19	27	4+	4+	4+	3+	±	±	0	0	0	0	0								0
19	31	4	0	0	0	0															0
19	31	19	4+	4+	4+	+	±	0	0	0	0	0	0								0
9/12	36	3	0	0	0	0	0	0													0
12	36	32	4+	4+	4+	3+	0	0	0	0	0	0	0	0							0
12	61	3	±	±	0	0	0	0	±	0	0	0	0	0							+
12	61	31	4+	4+	4+	4+	4+	3+	0	0	0	0	0	0							0
8/19	33	2	2+	±	0	0															0
19	33	17	4+	4+	4+	3+	2+	±	0	0	0	0	0								0
19	28	11	3+	±	±	0	0														0
19	28	26	4+	4+	3+	3+	0	0	±	0	0	0	0	0							0
19	T.W.H.	5	4+	3+	±	0															0
19	T.W.H.	18	4+	4+	4+	4+	4+	3+	0	0	0	0	0								0
4	7	12	3+	3+	2+	+	±								0	0	0	0	0		0
4	7	19	4+	4+	4+	3+	2+	+	0	0	0	0	0	0	0	0	0	0	0		0
19	6	11	4+	4+	4+	+	±														0
19	6	26	4+	4+	4+	4+	2+	±	0	0	0	0	0								0
19	15	14	4+	2+	+	±	0														0
19	15	29	3+	3+	+	±	0		0	0	0	0	0								0
19	52	12	4+	2+	+	±	±														0
19	52	20	3+	2+	+	0	0		±	0	0	0	0								0
19	16	13	3+	2+	±	±	0														0
19	16	29	3+	2+	±	±	±		±	0	0	0	0								0
7/31	H-4	24	4+	4+	2+	±	±	0													0
8/ 4	41	22	4+	4+	4+	4+	3+	3+	0	0	0	0	0	0	0	0	0	0	0	0	0
7/24	1	8	0	0	0	0	0														0
24	1	14	0	0	0	0	0														0
8/ 4	1	8	0	0	0	0	0	0	0	0	0	0	0	0							0
4	1	14	0	0	0	0	0	0	0	0	0	0	0	0							0

7/24	3	8	0	0	0	0	0	0											0
24	3	14	0	0	0	0	0	0											0
8/19	27	8	0	0	0	0													0
19	27	24	0	0	0	0	0	0	0	0	0	0	0	0					0
7/31	39	20	+	±	0	0	0	0											0
24	Immune Jap. B																		
	Hamster (Pool 1)		4+	4+	4+	2+	±												0
31	Immune Jap. B																		
	Hamster (Pool 2)		4+	4+	4+	4+													
8/ 4	Immune Jap. B																		
	Hamster (Pool 2)			4+	4+	4+									0	0	0		0
19	Immune Jap. B																		
	Hamster (Pool 3)		4+	4+	4+	4+	4+	±	0	0	0	0	0						0
9/12	Immune Jap. B																		
	Hamster (Pool 2)		4+	4+	4+	4+	4+	2+	3+	0	0	0	0	0					0
8/ 4	Immune St. Louis																		
	Mouse		±	0	0										4+	4+	4+	4+	0
4	Immune West.																		
	Equine																		
	G.P.								4+	4+	4+	4+	4+	3+	±	0	0	0	0
19	Immune West.																		
	Equine																		
	G.P.		+	0	0	0	0		4+	4+	4+	4+	4+						0
9/12	Immune West.																		
	Equine																		
	G.P.		2+	±	±	0	0	0	4+	4+	4+	4+	4+	4+					0

TABLE II.

Identification by Complement Fixation Reaction of Virus Isolated on Okinawa from Patient Suffering from Encephalitis.

Serum	Nakayama strain Jap. B antigen (Lyophilized) Final serum dilution 1 to: 4	8	16	32	64	128	Okinawa No. 53 antigen (Freshly prepared) Final serum dilution 1 to: 4	8	16	32	64	128	Normal mouse brain antigen (Freshly prepared) Final serum dilution 1 to: 4	8	16	32	64	128	Western equine antigen (Lyophilized) Final serum dilution 1 to: 4	8	16	32	64	128	Serum Control
Hamster—(Pool No. 1) vaccinated with Nakayama Jap. B virus	4+	4+	3+	±	±	±	4+	4+	4+	3+	+	±	0	0	0	0	0	0	0	0	0	0	0	0	0
Hamster—(Pool No. 2) vaccinated with Nakayama Jap. B virus	4+	4+	3+	±	±	±	4+	4+	4+	3+	±	±	0	0	0	0	0	0	0	0	0	0	0	0	0
Guinea pig vaccinated with West. equine virus	±	±	0	0	0	0	±	±	0	0	0	0	±	±	±	0	0	0	4+	4+	4+	4+	4+	4+	0

Heretofore, no sera from persons convalescing from an infection with Japanese B virus have been available for study by means of complement-fixation tests, so that it was not known whether a similar cross antibody reaction occurs in the sera of human beings. Table I shows that sera from 3 convalescent persons which fixed complement in the presence of Japanese B virus failed to do so with St. Louis virus. Furthermore, 15 sera which contained complement-fixing antibody against Japanese B virus showed no antibody against the virus of Western equine encephalitis. Two sera which were positive against Japanese B virus were negative against lymphocytic choriomeningitis virus, and 2 other such sera were negative against Eastern equine encephalitis.

The identification of Japanese B virus as the causative agent of the Okinawan outbreak of encephalitis by means of the serologic evidence described above was confirmed by the isolation of a virus from the brain of a patient (No. 53) dying of encephalitis. This virus was isolated by a group of medical officers of the U. S. Army and Navy (two of the authors of the present communication, L.T. and J.P., were in this group). A sample of this agent was sent to our laboratory on Guam. An antigen suitable for complement-fixation tests was prepared from brains of mice dying of infection with the third passage of this virus. As shown in Table II, the Okinawa virus antigen fixed complement in the presence of sera of hamsters immunized against the Nakayama strain of Japanese B virus, but no significant complement-fixation occurred when it was tested against serum of guinea pigs immunized against Western equine encephalitis virus. The virus isolated on Okinawa was, therefore, shown to be Japanese B virus by means of its specific complement-fixation reaction.

Summary. 1. By complement-fixation tests carried out with human sera, an outbreak of encephalitis which began on Okinawa in July, 1945, was promptly shown to be due to Japanese B virus. 2. The tests showed that at least 11 of 20 patients studied had suffered an attack of encephalitis caused by this virus. 3. None of 15 human sera which were positive against Japanese B virus showed complement-fixing antibodies against Western equine encephalitis virus, and a few such sera were negative when tested against the viruses of Eastern equine encephalitis, St. Louis encephalitis, and lymphocytic choriomeningitis. 4. A virus isolated from a patient on Okinawa suffering from encephalitis was identified by complement-fixation reactions as Japanese B virus.

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Observations on Antimicrobial Action of 2, 3-Dichloro-1, 4-Naphthoquinone, and its Reversal by Vitamins K.

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Since Ter Horst and Felix[1] have found a marked antifungal action of 2,3-dichloro-1,4-naphthoquinone, the idea occurred that this effect might be linked with the structural similarity of the compound to vitamin K, much as that of the sulfonamides is related to their analogy with the metabolite *p*-aminobenzoic acid. This view seemed probable since Kuhn *et al.*[2] had shown previously that 6,7-dichloro-9-ribityl-isoalloxazine had antimicrobial action which was reversible by riboflavin. The change involved in passing from the vitamin riboflavin to the inhibitor 6,7-dichloro-9-ribityl-isoalloxazine was the replacement of 2 methyl groups attached to

* With the technical assistance of M. L. Collyer.

1 Ter Horst, W. P., and Felix, E. L., *Ind. Eng. Chem.*, 1943, **35**, 1255.

2 Kuhn, R., Weygand, F., and Möller, E. F., *Ber. deutsch. chem. Ges.*, 1943, **76**, 1044.

TABLE I.

Amounts of 2,3-dichloro-naphthoquinone and of 2-methyl-naphthoquinone Required to Cause Half-maximal Inhibition of Growth of Various Microörganisms.

Organism	Amount for half-maximal effect	
	2,3-dichloro-naphthoquinone μg per cc	2-methyl-naphthoquinone μg per cc
Saccharomyces cerevisiæ	0.0017	0.23
Endomyces vernalis	0.015	0.28
Lactobacillus casei	20	20
Staphylococcus aureus	10	10
*Escherichia coli**	35	15
Hemolytic streptococcus H69D	35	40

* This organism was tested in the synthetic medium described earlier[6] for its growth. In the highly purified, but more complex medium used for the other bacteria in this work the quinones were less active, *e.g.*, 2-methyl-naphthoquinone was just detectable at 50 μg per cc.

a benzenoid nucleus with Cl atoms. If a similar type of structural alteration were applied to vitamin K, *i.e.*, if the 2 alkyl side chains were exchanged for Cl atoms, one would arrive at 2,3-dichloro-1,4-naphthoquinone. Therefore, since we had been investigating the types of structural change which would convert metabolites into specific inhibitory competitive analogs,[3] it was decided to determine whether the action of dichloro-naphthoquinone bore relationship to that of vitamin K. If the effects of the compound could be negated by the vitamin the actions of the two might conceivably be connected.

2,3-dichloro-naphthoquinone† was inhibitory to the growth of all microbial species tested. The amounts of the compound required to produce half-maximal effect are shown in Table I. It can be seen that there was very great variation in the susceptibility of various species to the agent. Bacterial forms examined were relatively resistant, while the yeasts were exceedingly sensitive to the quinone. The minute concentration needed to affect *Saccharomyces cerevisiæ* made this compound one of the most toxic known for this organism. The growth tests with bacteria were conducted in the highly-purified medium of Landy and Dicken,[4] and those with the yeasts in the synthetic medium previously described.[5] Growth was estimated turbidometrically in the usual manner,[6] and in the case of those species which produced acid, the turbidometric results were checked by estimating the final pH of the cultures. The quantity of agent which caused half-maximal inhibition of growth was determined by interpolation on a curve relating dose to response. It was necessary that the dichloroquinone be added after the various media were autoclaved, for when it was heated in the medium or even in water, its potency was reduced about 100 times, probably due to the conversion of the substance to 2-chloro-1,4-naphthoquinone.[7] To avoid heat-sterilization, a 2% solution of the dichloro-naphthoquinone in acetone was diluted with sterile water.

The toxicity of vitamin K in the form of 2-methyl-1,4-naphthoquinone was determined for each species of microörganism, for it is known that this compound, like many other quinones, is rather harmful to microörganisms. The data in Table I indicate the concentrations of this form of the vitamin which were necessary to cause half-maximal inhibition of growth under the same conditions as were used in the trials with the dichloroquinone. However, synthetic vitamin K_1 in contrast to 2-methyl-naphthoquinone was not inhibitory

[3] Woolley, D. W., *Science*, 1944, **100**, 579.

† The 2,3-dichloro-naphthoquinone was kindly supplied by Dr. G. L. McNew of the United States Rubber Company.

[4] Landy, M., and Dicken, D. M., *J. Lab. and Clin. Med.*, 1943, **52**, 106.

[5] Woolley, D. W., and White, A. G. C., *J. Exp. Med.*, 1943, **78**, 489.

[6] Woolley, D. W., *J. Biol. Chem.*, 1941, **140**, 453.

[7] Beilstein, F., *Handbuch der Organischen Chem.*, 1897, **3**, 372.

TABLE II.
Effect of 2,3-dichloro-naphthoquinone and of 2-methyl-naphthoquinone Singly and Together on the Growth of *Saccharomyces cerevisiæ.*

2,3-dichloro-naphthoquinone	2-methyl-naphthoquinone	Turbidity* of culture
μg per cc	μg per cc	
0	0	39
0.002	0	78
0.005	0	93
0.01	0	99
0.005	0.04	60
0.005	0.02	68
0.005	0.01	77
0.005	0.005	85
0.002	0.02	48
0.002	0.01	66
0	0.05	46
0	0.20	65

*Turbidity is expressed as per cent of the incident light transmitted by the culture when the uninoculated basal medium is considered to have 100% transmission.

to the growth of either *Lactobacillus casei* or *S. cerevisiæ.*

With *S. cerevisiæ* and with *Endomyces vernalis* the harmful effects of 2,3-dichloro-naphthoquinone were antagonized by vitamin K. Over a limited range of concentration this antagonism was competitive as can be seen from the data in Table II. However, the inhibition index[5] (the ratio between concentration of inhibitor and that of vitamin required to negate the action of the former) was 0.1, and therefore, a point was soon reached, as the concentration of dichloro-naphthoquinone was raised, beyond which it was not possible to reverse the inhibitory effect with the vitamin, for the concentration of the vitamin was then in the toxic range. With *E. vernalis,* it was barely possible to achieve complete counteraction of the effect of the analog for, since the dichloro-naphthoquinone was less poisonous than with *S. cerevisiæ,* the dosage of the vitamin K required for reversal was approaching the toxic level.

With the other species examined, no reversal of the action of dichloro-naphthoquinone was accomplished by additions of vitamin K either as 2-methyl-naphthoquinone or as vitamin K_1. This was not surprising, however, for the fact that the inhibition index was less than 1 for this pair of structural analogs indicated that the amount of vitamin needed to exert a reversing effect was beyond the toxic level. Since 20 μg of dichloro-naphthoquinone were needed for half-maximal inhibition of growth of *L. casei,* 200 μg of 2-methyl-naphthoquinone would be necessary to counteract the effect. However, 2-methyl-naphthoquinone was toxic at levels lower than this. If 2-methyl-naphthoquinone were not such a potent inhibitor of microbial growth it might be possible to reverse the action of dichloro-naphthoquinone in more species. Perhaps the failure to reverse the anti-microbial action of 3,3′-methylenebis-(4-hydroxycoumarin)[8] may have a similar explanation, for it is known that the inhibition index for this compound and vitamin K in rats is less than 1 just as in the case of the present example.[9]

With *S. cerevisiæ,* vitamin K_1 was about equal (on a molecular basis) in activity to 2-methyl-naphthoquinone in causing reversal of the effects of dichloro-naphthoquinone, and phthiocol (2-methyl-3-hydroxy-1,4-naphthoquinone) had 1/30 the potency. This is about the same relative potency of these 3 compounds when tested for their ability to cure vitamin K deficiency in chicks.

If dichloro-naphthoquinone acts in microbes by production of vitamin K deficiency, one would expect that it would cause signs of vitamin K deficiency in animals. Experiments were performed in which graded amounts of the substance were fed to weanling mice maintained on a highly purified adequate basal ration[10] minus vitamin K. The rate of

8 Goth, A., *Science,* 1945, **101**, 383.

9 Overman, R. S., Field, J. B., Baumann, C. A., and Link, K. P., *J. Nutrition,* 1942, **23**, 589.

10 Woolley, D. W., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 565.

TABLE III.
Effect of 2,3-dichloro-naphthoquinone on Growth and Survival of Weanling Mice Fed a Highly Purified Ration Free of Vitamin K.

Amt in diet, %	No. of animals	Deaths	Avg change in wt, g per wk
0	8	0	+5.0
0.1	4	0	+3.3
0.2	4	0	+1.4
0.5	14	7	—1.0

growth was diminished detectably when 0.1% of the diet consisted of the quinone, and when this was increased to 0.5%, half the animals died within 3 weeks (Table III). The mice which died did not, however, show signs of hemorrhage macroscopically as do mice fed 3,3′-methylenebis-(4-hydroxycoumarin). Furthermore, the prothrombin time of the diluted plasma of mice fed 0.5% of the quinone was not significantly greater than that of the controls or of normal mice. Finally, simultaneous administration of 2-methyl-naphthoquinone or of vitamin K_1 did not erase the toxic manifestations of 2,3-dichloro-naphthoquinone.

Since vitamin K has not been demonstrated in yeast, it may be that dichloro-naphthoquinone and the vitamin are just 2 foreign substances contending non-specifically in the yeast cells, which fall prey to either agent or to both, or to neither, depending on relative and absolute concentrations. However, vitamin K is known to exist in some microörganisms, and to be an essential for growth of the Johne's bacillus.[11] The concentration of the vitamin in yeast may be so small as to be undetectable by present methods, and yet sufficient to supply each cell with many molecules.

From the examples now at hand it would seem that the replacement of methyl groups attached to benzenoid nuclei in metabolites with Cl atoms can lead to inhibitory structural analogs which compete with these metabolites. More examples would be desirable to determine whether it is a type of change which is generally applicable.

Summary. 2,3-dichloro-1,4-naphthoquinone, an antifungal agent now in practical use, has been recognized as an analog of vitamin K. This substance has been found to be exceedingly toxic to yeasts and moderately harmful to the growth of bacteria. Its effect on yeast was reversed competitively by vitamins K over a limited range of concentration. While the effect on the growth of bacteria was not influenced by vitamin K in the form of 2-methyl-naphthoquinone, it was probable that this was due to the toxicity of the vitamin for these species.

[11] Woolley, D. W., and McCarter, J. R., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 357.

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The Porphyrin of Harder's Gland.

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The fact that choline poisoning stimulates the flow of brick red tears, designated as chromodacryorrhea,[1] has been reported and confirmed; however, the nature of the pigment, protoporphyrin[2] or blood,[3] is in dispute.

The presence of a porphyrin in Harder's

[1] Tashiro, S., and Stix, H., *Biol. Bull.*, 1935, **64**, 327.

[2] Barnard, R. D., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 254.

[3] Hodge, H. C., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 26.

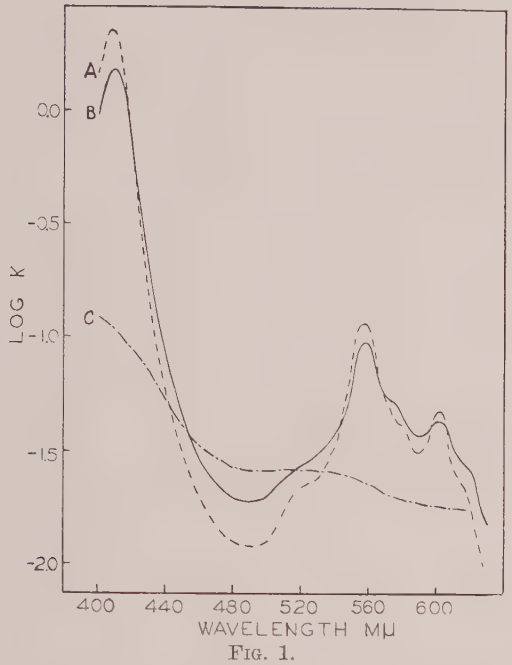

FIG. 1.

Spectrophotometric curves for the red pigment of "bloody" tears (A), extract of Harder's gland (B), and extract of rat blood (C).

gland in the rat and its excretion from that gland has been described.[4] On the basis of these observations, our attention was directed to the role of Harder's gland in chromodacryorrhea produced by choline poisoning. In the present study, young adult rats of both sexes were given intraperitoneal injections of choline chloride, about 200 mg in aqueous solutions. Convulsions and secretion of deep red tears were followed by death in each of about 20 rats.

Microscopic examination of the red tears revealed a field full of spherical, colorless, clear droplets among which were fragments of yellow-brown pigment. A source of ultraviolet light, a Corning ultraviolet filter and a glass optical system provided illumination in the near-ultraviolet. This light when directed through the condenser upon the microscopic field caused the pigment particles to fluoresce a brilliant fuchsia, whereas the spherical droplets remained dark.

Absorption spectra. A sample was prepared for spectrophotometric analysis as follows: The red tears from several animals were stirred with 2 cc of water and centrifuged at high speed. The turbid, nearly colorless supernatant liquid was decanted, leaving a dark red solid at the bottom of the tube. The red solid was washed by trituration and centrifuging with two 1-cc portions of distilled water. A small portion of the washed pigment was dissolved in 0.1 *N* hydrochloric acid. Centrifugation threw down a thin film of colorless material, leaving a clear, slightly pink solution that showed intense red fluorescence under the ultraviolet lamp. Determination of its absorption spectrum, using the Beckman quartz spectrophotometer, gave curve A in Fig. 1.

$$K = \text{Log} \frac{I_o}{I}$$

Comparison of the absorption curve of our red pigment with data published by Hans Fischer[5] (Table I) showed that our pigment is a protoporphyrin. This finding confirmed the observation of Barnard.[2]

TABLE I.
Comparison of Absorption Characteristics of Protoporphyrin and Pigment.

		Proto-porphyrin (5) ($m\mu$)	Pigment from red tears ($m\mu$)
I	Band width	607.6-597.3	606-596
	Band maximum	602.4	601
II	Band width	586.5-577.9	586-576
	Band max.	582.2	580
III	Band width	565.3-549.2	564-549
	Band max.	557.2	557

To determine whether hemoglobin might also be in the red tears, Dr. Alexander Dounce performed a pyridine hemochromogen test and examined a second sample of the tears for methemoglobin. These examinations were made with a hand spectroscope and in each case, bearing in mind the limits of the hand spectroscope, the absence of the characteristic bands showed that no appreciable amount of blood was present in the red tears.

Gland pigment. Two pairs of fresh Harderian glands were prepared for the spectro-

4 Derrien, E., and Turchini, J., *C. R. de la Soc. de biol.*, 1924, **91**, 637.

5 Fischer, H., Abderhalden, *Handbuch der biologischen Arbeitsmethoden*, 1936, **1**, 169.

photometer by washing with physiological saline to remove superficial blood. The glands were dried on filter paper and ground in a mortar with a pinch of sand and a little quick-setting plaster of paris. The fine, dry powder was ground with 2 portions of ether to remove fat. It was then ground with 2 cc of 0.1 *N* hydrochloric acid. The acid solution was centrifuged and the absorption spectrum of the clear, pink supernatant solution was determined, giving curve B in Fig. 1, which is very much like the curve of the pigment from the tears. This solution also showed intense red fluorescence under the ultraviolet lamp.

A few drops of fresh, normal rat blood in water was put into a mortar and treated in the same manner as the gland had been. The extract thus obtained had an absorption spectrum which is shown by curve C in Fig. 1. The porphyrin which was extracted from the red tears and from Harder's gland cannot be extracted from blood.

Unilateral extirpation. Under ether anesthesia, Harder's gland was removed from the right ocular orbit in 11 rats. After 2 weeks, the right eyes of these rats seemed normal except for their sunken appearance. Toxic doses of choline were injected intraperitoneally, and in every animal red tears welled up into the left eye but not in the right eye. In at least 6 of the animals a large pool of colorless tears could be detected in the right eye, indicating that in these animals, at least, lachrymal ducts and glands remained intact. The pigment in the red tears induced by choline poisoning undoubtedly has its origin in the glands of Harder.

Gland stimulation. Some interesting observations were made with reference to the stimulation of Harder's gland. If the common carotid artery and the external and internal jugular veins were ligated on one side of the rat, then the chromodacryorrhea following intraperitoneal choline injection was just perceptible on the ligated side but profuse on the normal side. Injection of choline into the common carotid produced highly pigmented tears, of a chocolate rather than brick red color. Careful observation revealed that the tears first appeared on the same side as the injection. When such an injection is made in a rat not more than a few minutes dead, the red tears appear only on the same side as the carotid which is injected. Choline injected topically behind the eyeball and into the orbit also produces chromodacryorrhea. These observations indicated that the choline affected the Harder's gland directly rather than through some central mechanism. Electrical stimulation of the gland with an inductorium produced red tears from that eye, but intracranial stimulation of the fifth and seventh nerves had no effect.

Histological observations. Under light ether anesthesia the right Harderian gland of a rat was electrically stimulated to produce red tears, and then both glands were removed and fixed in 10% formalin. Frozen sections were prepared and the uncleared, unstained sections were put on slides (in either water or glycerine) and gelatin-sealed under coverslips. Microscopic examination showed that many of the collecting ducts were filled with yellow-brown pigment. The number of filled ducts was much greater in the control gland than in the stimulated gland. With near-ultraviolet illumination, all of the tissue fluoresced a brilliant fuchsia. The control sections seemed to fluoresce somewhat more than the sections of stimulated gland but, since some sections of the latter equaled the former in brilliance, no definite difference can be reported. The pigment showed no fluorescence. At first this was attributed to its thickness and opacity; but even when illuminated from above, these areas remained dark. These observations were made of the sections as they appeared immediately after their preparation.

After the sections had been prepared for about 3 weeks they were again examined with the fluorescence microscope. The brilliance of the fluorescence in areas where there were no pigment-filled tubules had faded markedly. The cells surrounding the pigment fluoresced as much as, or more than, they had at first; and some of the thinner (as judged by their appearance in white light) pigment areas showed a deep ruby-red fluorescence. Apparently the pigment in the tubules has undergone some change while standing.

Summary. A pigment from Harder's gland

has been identified as a protoporphyrin. It is this pigment and not blood that appears in choline-induced chromodacryorrhea.

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Serological Differentiation of Primary Atypical Pneumonia from Virus Pneumonia of the Psittacosis Group.*

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Despite the widespread occurrence of agents of the psittacosis-lymphogranuloma group in birds and mammals, there is now definite evidence that few cases of virus pneumonia are caused by psittacosis or related agents, while many atypical pneumonias may be caused by a new filterable virus recently transmitted to chick embryos, cotton rats, and hamsters.[1] Eaton and Corey,[2] using complement fixation tests with the virus of meningopneumonitis propagated in allantoic fluid and the virus of lymphogranuloma venereum grown in yolk sacs of chick embryos, found that among 70 patients with pneumonitis of unknown etiology, 10 to 15% showed an increase in complement-fixing antibodies for the psittacosis group. Isolation of a psittacosis-like virus was successful in only 10 of over 250 specimens of sputum or lung inoculated intranasally into mice or cotton rats.[2,3] In a series of 45 cases Smadel,[4] using agar slant tissue cultures of psittacosis virus as antigen, demonstrated a significant rise in complement-fixing antibodies in 10 cases, or about 22%. The virus was isolated from 2 of these patients. Workers in Francis' laboratory,[5] using an antigen from mouse lung infected with meningopneumonitis virus and refined by differential centrifugation, obtained a significant increase in titer (fourfold or greater) in only 1 case among 57 cases of atypical pneumonia. In the same laboratory, psittacosis-like viruses were isolated from the sputum or throat washings of 5 of 20 patients by inoculation of Java rice birds and subsequent intranasal passage in mice or by passage in mice alone. The relation of these findings to the etiology of atypical pneumonia was not clear, since none of the 5 patients had any increase in complement-fixation titer for meningopneumonitis associated with their illness. In a recent article Dingle[6] states that in the army the diagnosis of psittacosis or ornithosis was not once established in a series of more than 500 patients with atypical pneumonia and other respiratory infections. The method of testing was not stated.

During an epidemic of 19 cases of pneumonitis in the bayou region of Louisiana, a virus similar to the "psittacine group" was isolated from the throat washings of 3 of 4 patients studied and from the lungs at autopsy of 2 of these patients.[7] This outbreak seems to have been quite different in its general characteristics from primary atypical pneumonia. In other publications since 1940 psitttacosis-like viruses were identified as the

* The studies and observations on which this paper is based were supported by the International Health Division of The Rockefeller Foundation, in cooperation with the California State Department of Public Health.

1 Eaton, M. D., Meiklejohn, G., and van Herick, W., *J. Exp. Med.*, 1944, **79**, 649; *J. Exp. Med.*, 1945, **82**, 317, 329.

2 Eaton, M. D., and Corey, M., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 165.

3 Meiklejohn, G., Beck, M. D., and Eaton, M. D., *J. Clin. Invest.*, 1944, **23**, 167.

4 Smadel, J. E., *J. Clin. Invest.*, 1943, **22**, 57.

5 Francis, T., Jr., quoted by Dingle, J. H. *et al.*, *Am. J. Hygiene*, 1944, **39**, 269.

6 Dingle, J. H., *Bull. N. Y. Acad. Med.*, 1945, **21**, 235.

7 Olson, B. J., and Larson, C. L., *Pub. Health Rep.*, 1944, **59**, 1373.

causative agents in small groups of 2 to 6 cases, and where large numbers of cases of atypical pneumonia were analyzed only single blood specimens were tested by complement fixation. Additional papers contain details of the isolation of psittacosis-like viruses from birds or mammals without convincing evidence of the etiological relation of these agents to virus pneumonia in man.

The results of complement fixation tests with certain antigens prepared from rodent lungs indicate that the test for the psittacosis group in primary atypical pneumonia may not always be specific. Thomas and his associates[8] obtained in 14 of 35 cases of atypical pneumonia an increase in complement-fixing antibodies to suspensions of mouse lungs infected with the pneumonia virus of mice,[9] and sera of 5 of the patients also reacted with dissimilar antigens prepared from the viruses of influenza, cat pneumonitis, and meningopneumonitis. In our laboratory,[1,10] increases in complement-fixing antibodies were observed in 8 of 23 cases of atypical pneumonia with an antigen prepared from the sedimentable tissue components of hamster lungs infected with a virus isolated from cotton rats which is unrelated to the virus of atypical pneumonia or to any of the agents mentioned above.

Because of the occurrence of these apparently non-specific reactions in some cases of atypical pneumonia, differentiation of psittacosis and ornithosis from primary atypical pneumonia by the complement fixation test alone is somewhat uncertain. The development of a neutralization test with the virus recently isolated from patients with atypical pneumonia[1] facilitates such differentiation, as is shown in the present paper.

Materials and Methods. Complement fixation tests for the psittacosis group were performed with allantoic fluid antigens of the virus of meningopneumonitis and yolk sac antigens of the virus of lymphogranuloma venereum, as previously described.[11] One modification was introduced; the yolk sac antigen was extracted 3 times with ⅔ volume of ether, the ether being pipetted off after centrifugation.

Neutralization tests with the virus of primary atypical pneumonia (strain De) were done according to the published method[1] using serial dilutions of serum and 10% suspensions of chick embryo lung, trachea and amniotic membrane containing the virus. Titers were recorded as the highest final dilution of serum which completely prevented the appearance of pulmonary lesions in hamsters inoculated intranasally with the serum-virus mixtures, when 75 to 100% of the animals inoculated with virus plus normal horse serum had lung lesions.

Agglutination tests were done with the indifferent streptococcus No. 344 isolated by Thomas and his associates,[12] using the method described in the original report.

Cold agglutination tests were done with 1% suspensions of type O human erythrocytes, according to the technic previously described.[13] In the tests for complement fixation, streptococcal agglutination, and cold agglutination the titers were expressed in terms of the original serum dilutions before adding the reagents. In the agglutination tests titers less than 10 were considered not significant and were recorded as zero.

Results. Table I contains the data derived from complement fixation, neutralization, and agglutination tests with sera of 11 patients with primary atypical pneumonia, all of whom showed increases in neutralizing antibodies to the virus of atypical pneumonia, as indicated in the third column of the table. The virus was demonstrated in the sputum of 3 of these patients by inoculation of gradocol filtrates into the amnion of chick embryos[1] and subsequent passage to hamsters. Cold agglutinins

8 Thomas, L., Curnen, E. C., Mirick, G. S., Zeigler, J. E., and Horsfall, F. L., Jr., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 121.

9 Horsfall, F. L., Jr., and Hahn, R. G., *J. Exp. Med.*, 1940, **71**, 391.

10 Eaton, M. D., unpublished observations.

11 Eaton, M. D., Martin, W. P., and Beck, M. D., *J. Exp. Med.*, 1942, **75**, 21.

12 Thomas, L., Mirick, G. S., Curnen, E. C., Zeigler, J. E., and Horsfall, F. L., Jr., *Science*, 1943, **98**, 566.

13 Meiklejohn, G., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 181.

TABLE I.
Cases of Primary Atypical Pneumonia with Increases in Virus-Neutralizing Antibodies.

Case initials	Serum, days after onset	Serological titers: Neutralization virus of atypical pneumonia	Agglutination streptococcus No. 344†	Cold agglutination†	Complement fixation psittacosis group‡
Ta	4	2	0	10	4a
	31	8	0	80	4a
Ha	7	<2	0	10	8a
	20	4	10	320	8a
Yo*	4	<2	—	0	—
	25	64	40	640	4b§
Ex	2	4	0	—	32a
	30	32	0	—	32a
Mu*	4	<2	0	0	—
	18	8	10	1280	0b§
Bu*	4	<2	0	0	0a
	25	32	0	160	0ab§
Hr	6	<4	10	0	0a
	12	16	40	160	0a
Bt	5	<2	0	10	0a
	17	8	80	80	0ab
Do	5	<4	0	10	—
	25	16	20	160	0b§
Sc	9	4	20	10	0a
	19	16	20	80	0a
Pr	6	<2	0	10	0a
	26	8	0	40	0a

* Virus of primary atypical pneumonia isolated from sputum.
† 0 = titers of less than 10. — = not done.
‡ a = complement fixation with group antigen lymphogranuloma venereum.
b = complement fixation with group antigen meningopneumonitis.
§ Developed nonspecific complement fixation with hamster lung (see text).

appeared in 10 of the sera and 7 had agglutinins for the indifferent streptococcus in titers ranging from 10 to 80. It may be seen in the last column of Table I that no change in complement-fixing antibodies for the psittacosis group was found in 8 cases. In the remaining 3, only the convalescent sera were tested and all had titers below a significant level. Sera of four of the patients listed in Table I, Yo, Mu, Bu, and Do, gave nonspecific complement fixation with the sedimentable components† of normal hamster lung, and also of hamster lung infected with the agent W2 from cotton rats.[1] In these 4 cases the reaction went to a higher titer in the convalescent serum than in the acute phase serum.

In Table II are presented the serological results in 10 cases of virus pneumonia in each of which the cause was attributable to one of the strains belonging to the psittacosis group. From the sputum of 4 of these patients a psittacosis-like virus was isolated. As discussed in a previous report[3] the virus in cases Bc and Pr was identified as the SF strain, in case Cr as a strain of meningopneumonitis, and in case Sp as a strain of pigeon ornithosis. In 8 cases an increase in complement-fixing antibodies to the psittacosis group was demonstrated, as shown in the last column of the table. In the case Bc a rise in antibodies had been demonstrated with serum specimens taken on the 8th and 17th day of illness,[3] but these were no longer available at the time of the present study. In the remaining 2 cases only convalescent specimens were available. The patient Mg was a nurse who was presumably infected with the SF strain at the same time as 2 other nurses from whom this strain

† This material was obtained by centrifuging suspensions of lung first at 2,000 R.P.M. for 20 minutes in a horizontal head, then centrifuging the supernatant at 5,000 R.P.M. for one hour in an angle head. The 5,000 sediment after washing was used for complement fixation at 4°C for 18 hours.

TABLE II.

Cases of Virus Pneumonia with Increases in Complement-Fixing Antibodies to the Psittacosis Group.

Case initials	Serum, days after onset	Serological titers: Neutralization virus of atypical pneumonia	Agglutination streptococcus No. 344†	Cold agglutination†	Complement fixation psittacosis group‡
Wi*	2	8	0	0	8ab
	13	8	0	0	32ab
Ro	7	128	20	0	4a
	17	128	20	0	64a
Ho	6	<4	0	0	4a
	30	<4	0	0	64a
Bc*	pre	16	0	—	—
	8 mo.	16	0	—	16ab§
Cr*	pre	4	—	—	0b
	30	4	0	—	32ab
Pr*	pre	—	—	—	4b
	21	<4	0	—	32ab
Me	8	<4	0	—	4ab
	45	<4	0	—	16ab
St	9	—	—	—	8ab
	41	<4	0	—	32ab
Sp*	23	<4	0	—	32b
Mg	30	<4	0	—	32b

* Virus of psittacosis group isolated from sputum.

† 0 = titer of less than 10.

‡ a Complement fixation with group antigen lymphogranuloma venereum.

b Complement fixation with group antigen meningopneumonitis.

§ Increase in complement fixation titer was demonstrated with intermediate serum specimens.

was isolated,[14] but the only laboratory evidence of infection in case Mg was the high complement fixation titer. In those cases in which antigens from both meningopneumonitis and lymphogranuloma vereum were used the titers of a given serum with each antigen were the same or differed by only a twofold dilution.

In 6 of the cases in Table II paired serum specimens were available for neutralization tests with the virus of atypical pneumonia and no increase in antibodies to this virus was found. In the remaining cases the titers of the convalescent specimens were less than 4, indicating that the illness caused no significant response of neutralizing antibodies. The sera from 5 patients, Bc, Pr, Me, St, and Mg, had been stored at 4°C for 3 to 4 years at the time the neutralization tests were done. It is known, however, that the virus neutralizing antibody withstands storage for at least 2½ years at 4°C, and antibodies without change in titer were found in one 4-year-old serum (Bc in Table II).

[14] Eaton, M. D., Beck, M. D., and Pearson, H. E., *J. Exp. Med.*, 1941, **73**, 641.

Agglutinins for the indifferent streptococcus No. 344 were found in one of the 10 cases presented in Table II. In this case (Ro) there was no change in the agglutination titer and the initial level of neutralizing antibodies for the virus of atypical pneumonia was high. It is possible, therefore, that this patient, who was a nurse, may have been infected with the virus of atypical pneumonia some weeks or months before the illness which produced the rise in psittacosis-group antibodies. No cold agglutinins were found in the sera of three patients from whom fresh specimens were available for testing.

Discussion. From the available data it appears that the complement fixation test is a reasonably reliable indicator of infection with a virus of the psittacosis group when a definite increase in antibodies associated with illness is demonstrated and when precautions are taken to exclude the fixation with normal or pathological tissue components such as those found in rodent lungs infected with unrelated agents.[1,8,10] The cold agglutination and the non-specific complement fixation may be considered as serological reactions of a hetero-

genetic nature which frequently occur with primary atypical pneumonia, but are not necessarily limited to this disease. Many patients with atypical pneumonia show an increase in virus neutralizing antibodies without development of cold agglutinins or the other reactions. Evidence for the specificity of the virus neutralization in atypical pneumonia has been presented in this and other publications.[1] Agglutination of the indifferent streptococcus seems to occur with greater frequency in primary atypical pneumonia than in virus pneumonias of the psittacosis group. This finding suggests a possible specific association, at least in certain cases, of the streptococcus and virus in the pathogenesis of the disease.[15]

Data presented elsewhere[1] indicate that the virus of atypical pneumonia isolated and propagated in chick embryos is distinct in respect to host range, pathogenicity, and other properties from viruses of the psittacosis-lymphogranuloma group. The results presented in this paper show that these agents are also antigenically distinct as judged by complement fixation and neutralization tests on human sera.

Summary. In 11 cases of primary atypical pneumonia with increases in neutralizing antibodies for the virus propagated in chick embryos no significant development of complement-fixing antibodies for agents of the psittacosis group were found. Ten of the patients had cold hemagglutinins and 7 showed significant titers of agglutinins for the indifferent streptococcus No. 344. In an additional 10 cases of virus pneumonia where the presence of an agent of the psittacosis group was demonstrated by virus isolation or complement fixation, no significant development of neutralizing antibodies for the virus of atypical pneumonia occurred. The serum of one of the latter 10 patients showed streptococcal agglutination at a dilution of 1:20 without change in titer.

Dr. Gordon Meiklejohn collected many of the specimens and compiled much of the clinical data. The assistance of Miss M. Dorthy Beck, Miss Marilla Corey, Miss V. Lee Hanford, Miss Marjorie Hunt, and Mr. William van Herick is gratefully acknowledged.

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15145

Experimental Intestinal Myiasis in Man.*

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The possibility of a true intestinal myiasis has been much discussed.

It was demonstrated that, when swallowed, the maggots of many different species may cause violent abdominal distress, vomiting, diarrhea and even systemic reactions. It is not quite clear however if these disturbances are caused by the accidental presence of dipterous larvæ and will clear up after their rapid elimination, or if some species of maggots can adapt themselves to intestinal environments, survive for a certain time, even multiply and cause a true intestinal myiasis.

A few observations seem to indicate that such possibility exists. Herms and Gilbert[1] described a case in which a woman apparently suffered from intestinal myiasis that had extended for many years, Thebauld[2] recovered living dipterous larvæ from the stool of a child suffering from typhoidal affection. In

* This study was made possible through the facilities of the Governmental Medical Services of the Belgian Congo.

1 Herms, W. B., and Gilbert, Q. O., *Ann. Int. Med.*, 1933, **6**, 941.

2 Thebauld, V., *Arch. Parasit.*, 1901, **4**, 353.

many other cases different species of dipterous larvæ such as *Musca domestica, Fannia canicularis, Fannia scalaris,* Muscina, Sarcophaga, Calliphora, Eristalis, *Apiochaeta scalaris, Piophila casei,* etc., some of them dead, some alive, were recovered from stools or vomitus, but none of these observations seem to prove that they dealt with a true intestinal myiasis.

Description of the Experiment. Sixty human volunteers of Katanga, Belgian Congo, were fed living maggots of *Musca domestica,* Calliphora, and Sarcophaga. All larvæ were administered in large gelatin capsules on a fasting stomach, with 2 glasses of water. Each man received 20 larvæ of a single species. The reactions that followed can be summarized in 5 groups.

Group 1. In 18 cases vomiting occurred very shortly after administration of larvæ and the totality of maggots were recovered from the vomitus, some still in capsules, most of them free, with few exceptions all larvæ were alive. Vomiting subsided immediately after elimination of larvæ.

Group 2. In 12 cases gastro-intestinal disturbances occurred a few hours after ingestion. Late vomiting brought a few dead larvæ, while many dead maggots were recovered from diarrheal stools. The symptoms cleared 24 hours after administration.

Group 3. In 16 cases no vomiting occurred, but diarrhea following slight intestinal distress brought many larvæ, all dead, in the first 24 hours. All symptoms disappeared after 48 hours.

Group 4. In 4 cases, no vomiting, but severe nausea and abdominal cramps with diarrhea occurred in the first 24 hours. Many dead and occasional living maggots were recovered from the stools in the first 36 hours. All symptoms disappeared after 48 hours.

Group 5. In 10 cases no disturbances were caused by the larvæ. Many dead maggots were recovered from the stools during the 48 hours that followed the ingestion.

In all 5 groups the 3 dipterous species were represented, thus indicating that different reactions were dependent rather upon the individuals than upon the species of larvæ administered.

Of the 60 men, 4 remained under observation only 3 months, 22 for 6 months, and 34 could be observed for 12 months. Stools of all men were examined regularly once a month and on each occasion when gastrointestinal symptoms occurred. No dipterous larvæ were found in any of them. The occasional gastro-intestinal disturbances disappeared spontaneously in all cases, the percentage of which was not higher than in the local population not under experimentation.

Discussion. Causey[3] who fed larvæ of a number of species to dogs, never observed any of the larvæ survive in the stomach for longer that 3 hours, and Chandler[4] believes that in cases when larvæ survive the passage through the gastro-intestinal tract, it is associated with low HCl in the stomach or a particularly rapid passage into the intestines. Since in our experiment larvæ were administered on a fasting stomach to avoid high acidity, this condition together with accelerated peristalsis due to the presence of maggots, might have permitted the passage of a few living larvæ.

Komarek,[5] who conducted a series of experiments with dipterous larvæ, has shown that larvæ need free oxygen, but this seems not to be true in the case of gastrophilus, a parasite of the alimentary canal of horses, where larvæ live until mature and ready to pupate.

In the case of Herms and Gilbert, not only a survival of larvæ is noticed, but this case implies intestinal pedogenesis, since the duration of the recovery of maggots was lasting far beyond the normal time of larvæ evolution of Calliphora, Lucilia, and Sarcophaga, and it is difficult to understand such abnormal multiplication.

Conclusions. From 60 volunteers fed with living maggots of *Musca domestica,* Calliphora, and Sarcophaga, under conditions to avoid their destruction in the stomach, only 10 failed to have symptoms of gastro-intestinal disturbance.

In 50 cases men had nausea, vomiting, intestinal cramps and diarrhea together or as

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4 Chandler, A. C., *Introduction to Human Parasitology,* 1936, 6th ed.

5 Komarek, J., *Mém. du Musée Roy. d'Inst. Nat. de Belg.,* 1936, **3**, 23.

separate symptoms, but all symptoms disappeared within 48 hours following the elimination of the larvæ, of which only a few were found alive in the vomitus and stools.

These findings seem to indicate that though temporary gastro-intestinal distress may follow the ingestion of such dipterous larvæ as *Musca domestica,* Calliphora, and Sarcophaga, they do not produce a true intestinal myiasis in man.

15146 P

Impedance Changes in the Cerebrum Following Concussion.*

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Since cellular injury is associated with impairment of cellular surface membranes (Osterhout[1]), it seems reasonable to surmise that cerebral concussion produces similar changes in the cerebrum. The electrocorticogram does not permit definite conclusions (Walker, Kollros, and Case[2]) regarding the cellular surface films, since other factors may affect it. We approached this problem by measuring the A.C. impedance of the cerebral hemispheres in 14 cats *in vivo* and in 50 guinea pigs immediately post mortem, at a low frequency (l = 570 cycles per second) and a high frequency (h = 5100 cycles), and computing

$$\Delta = \frac{(\text{Conductivity}_h\text{-Conductivity}_l \times 100}{\text{Conductivity}_l}.$$

Δ is a measure of the polarizability. In agreement with Gildemeister's[3] theory, we have given experimental evidence on artificial membranes and frog skin (Spiegel and Spiegel-Adolf[4]) that decrease of polarizability is associated with increase of electrolyte permeability. Acceleration concussion (Denny-Brown and Russell[5]) was produced by means of a pendulum, under nembutal anesthesia.

Results. In control experiments on cats Δ remained constant for at least 2 hours when the experimental conditions were identical except for omission of the blow. Blows inducing signs of concussion, typical changes of blood pressure, transitory apnea, loss of the corneal-, light-, pinna reflex, induced a mean decrease of Δ of 23.4 $\pm$ 1.6%. Usually it became most marked within ½-1 hour after the blow. Then a slow rise of Δ from the minimum began to develop. However, 4 hours after the blow a definite decrease of Δ was still noticeable. These changes of Δ did not appear if the pendulum struck a dead animal. It should be emphasized that the decrease of Δ developed independently of the fluctuations of conductivity, which were too irregular to permit definite conclusions.

Since in these experiments the skull had to be trephined for introduction of the electrodes before concussion, these measurements were repeated in guinea pigs concussed with the skull intact. Δ was measured only following the blow. After decapitation in various stages of concussion (see below), the head, with the cerebrum exposed, was placed in an incubator (38°C). In Group I (13 nonconcussed, but otherwise identically treated animals; 10% nembutal .2 cc per lb) the mean Δ was

* Aided by a grant from the John and Mary R. Markle Foundation.

1 Osterhout, W. J., *Injury, Recovery, and Death in Relation to Conductivity and Permeability,* J. B. Lippincott Co., Phila., 1922.

2 Walker, A. E., Kollros, J. J., and Case, T. J., *J. Neurosurg.,* 1944, **1**, 103.

3 Gildemeister, M., *Handb. d. norm. u. path. Physiol.,* 1928, **8**, II, 693.

4 Spiegel, E., and Spiegel-Adolf, M., *Am. J. Psychiat.,* 1936, **92**, 1145; *Am. J. Physiol.,* 1941, **133**, 459; Spiegel-Adolf, M., *J. Gen. Physiol.,* 1937, **20**, 695; Spiegel-Adolf, M., and Spiegel, E., *J. Colloid Science,* in press.

5 Denny-Brown, D., and Russell, W. R., *Brain,* 1941, **64**, 93.

10.84 ± .16† (M_1‡). In Group II, 11 animals were subjected to severe blows (energy 21.3-25.7 foot-pounds, velocity of striking piece 23.8-26.2 feet per second) and decapitated 1-2 minutes after the blow during apnea and loss of the corneal-, pinna-, and righting reflexes; the mean Δ was 8.91 ± .27 (M_2). Groups III to V were subjected to 1-3 slighter blows (energy 3.2-17.0 foot-pounds, velocity 10.5-21.4 feet per second), which were however still sufficient to produce typical concussion. In Group III, 12 rats were decapitated 2-8 minutes after the blow; the mean Δ was 11.21 ± .23 (M_3); Group IV, 7 rats, decapitated 10-60 minutes after the blow, showed a mean Δ = 9.52 ± .18 (M_4); Group V, 7 rats, decapitated 2-5 hours after the blow had a mean Δ = 11.05 ± .26 (M_5). The t values§ for the differences M_1-M_2, and M_1-M_4 were 5.6 and 3.3 respectively, *i.e.*, above the values necessary to consider these differences significant, while M_3 and M_5 lay within the normal variability. Thus only severe blows produced a definite decrease of Δ within the first minutes following the blow. Following slight, but still concussive blows a decrease of Δ was not demonstrable within the first minutes (Group III), but only 10-60 minutes following the blow (Group IV), at a stage when the apnea and disturbance of reflexes had long subsided. Group V shows that Δ returned to normal several hours after the blow.

† Figure after ± equals probable error of the mean.

‡ This value is not comparable to that previously obtained by us (*J. Nerv. Ment. Dis.*, 1941, **93**, 750) due to differences of the electrodes.

§ t = difference between the means divided by the standard error of the difference.

Analysis. These changes of Δ were also observed in cats kept under artificial respiration, so that cerebral anoxia due to apnea is, at most, only a contributory factor. Decrease of Δ developed with as well as without initial rise of femoral blood pressure. However, the subsequent fall of blood pressure below the initial level may be at least partly responsible for the impairment of the cells. Accumulation of lactic acid probably is of little importance, since artificial acidosis lowers the polarizability of the brain only slightly if at all (Spiegel and Spiegel-Adolf[6]). Similarly to some other postconcussional changes (chromatolysis, Windle, Groat, Fox[7]), appearance of nucleosides in the cerebrospinal fluid (Spiegel-Adolf, Henny, Wycis, and Spiegel[8]), increase of brain volume (White, Brooks, and Goldthwait[9]), the injury of the cellular surface membranes indicated by the decrease of polarizability becomes manifest or reaches its maximum at a stage in which the transient symptoms immediately following the concussion have already disappeared. The impairment of the cellular surface films by itself apparently does not suffice to explain the genesis of these symptoms. Rather it may be of importance in the development of symptoms appearing some time after the blow (confusion, headache, vertigo, etc.).

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7 Windle, W. F., Groat, R. A., and Fox, C. A., *Surg., Gynec., and Obstet.*, 1944, **79**, 51.

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15147

Growth of Corpus Luteum in Rats During Pregnancy and Following Injections of Testosterone.

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The corpus luteum increases in size during pregnancy in many species including man. The period of pregnancy during which the maximum size is reached varies among species[1] and corresponds, in part at least, to the length of time during which a functioning corpus luteum is the main source for progesterone. Experimental work during the past 20 years has shown that certain of the sex hormones when injected into suitable animals may produce an increase in the size of the corpus luteum comparable to that occurring spontaneously during pregnancy.[2,3,4] The experimental observations suggested that these hormones, particularly the estrogens, might contribute to the enlargement of the corpus luteum of pregnancy.[5]

During the past years, changes were noted in our rats following the injection of testosterone propionate during late estrus.[6] These changes included enlargement of the corpora lutea, extensive mucification of the vaginal epithelium, proliferation of ducts and alveoli in the mammary glands, and transformation of the endometrial stromal cells to decidua-like cells following proper traumatization of the uterus. Various phases of these experiments have been reported previously. The observations emphasized not only the importance of the corpus luteum with respect to certain effects of testosterone upon the rat, but also the similarity of the experimentally induced changes to the pregnant state. Hence, it became important to know to what extent the corpora lutea differed from or resembled those of pregnancy following injections of testosterone.

Data covering the growth and composition of the pregnancy corpus luteum were necessary first of all. In the literature such information concerns measurements of various diameters after fixation,[3,5,7,8,9] histochemical studies and extraction methods for the lipids,[7,10–14] and ascorbic acid.[15] The results may be briefly summarized by stating that neither lipid nor ascorbic acid content can quantitatively account for the increase in the size of the corpus luteum. Furthermore, differences in the species and methods make it difficult to gain an understanding of the changes within the corpus luteum during its growth and regression.

Therefore we decided to analyze the corpus

* Agnes Lemme Schilling Research Fellow.

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13 Weinhouse, S., and Brewer, J. I., *J. Biol. Chem.*, 1942, **143**, 617.

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TABLE I.
Groups and Periods of Experiments.

Pregnancy			Test. prop. 0.5 mg/day			Test. prop. 2.5 mg/day		
Day exp.	No. rats	No. of corp. lut.	No. rats	No. of corp. lut.	Total dose, mg	No. rats	No. of corp. lut.	Total dose, mg
Controls	65	646						
6			6	66	2.5			
11	19	184	25	238	5.0	16*	149	25.0
14	5	53						
16	15	171	17	162	7.5	10*	97	37.5
18	5	53						
21	16	171	42*	406	10.0	15*	159	50.0
24	4	42						
26	6	65	19*	198	12.5			
31	6	61	9*	87	15.0			
36	6	55						
Total	82	855	118	1157		41	405	

* The groups include rats with imperfect response.

luteum at various periods of pregnancy and to compare the results with those which might be obtained at corresponding periods of testosterone treatment. The present report describes the findings of the changes in growth and composition of the corpus luteum of the rat in these two conditions.

Material. A total of 306 female rats was used. They belonged to the Stanford strain of albino rats started from the University of Chicago stock in 1903. The strain had been observed particularly with respect to the reproductive phenomena during the past 3½ years. Because correct timing of the first injections with respect to the stage of the estrous cycle was of importance, many observations of the frequency and duration of the cycles were analyzed. A high degree of uniformity was found; 2966, or about 83%, of 3576 cycles lasted 4 to 5 days.

Methods. The vaginal estrous cycle of all animals was followed for 28 days after which all rats with cycles shorter than 4 and longer than 6 days were discarded. The age of the animals ranged from 90 to 150 days at the start of the experiment.

The control values were obtained from 65 rats which were killed within 24 hours after vaginal estrus had disappeared.

Eighty-two rats were used for the analysis of corpora lutea during pregnancy. They were placed with males during proestrus and the presence of spermatozoa in the vagina was assumed to indicate the onset of pregnancy. The animals were then housed individually and killed as indicated in Table I. Those rats which were reserved for the post partum period were permitted to nurse and the litter was uniformly adjusted to 6 sucklings.

The rats in which the corpora lutea were analyzed after hormone injections received the initial treatment during late estrus after cornified cells had been present in the smear for at least 15 hours. Two dosage levels were used. To 118 rats testosterone propionate† was given subcutaneously in daily doses of 0.5 mg in 0.1 cc of sesame oil over the periods given in Table I. This dosage was previously found to be the smallest amount which in the majority of our animals would result in persistent functioning corpora lutea and positive decidual reactions after uterine traumatization.[16] This experimental procedure was used in the 42 rats injected for 11 and 16 days respectively. Daily doses of 2.5 mg in 0.1 cc of sesame oil were given to 41 rats and they were killed at intervals which are indicated in Table I.

The animals were killed with ether 24 hours after the last injections and the corpora lutea individually dissected out under the microscope using the moist peritoneal surface as the operating field. As fast as possible, the

† Testosterone propionate (Perandren) was generously supplied by the Ciba Pharmaceutical Products, Inc., Summit, N.J.

16 Fluhmann, C. F., and Laqueur, G. L., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 223.

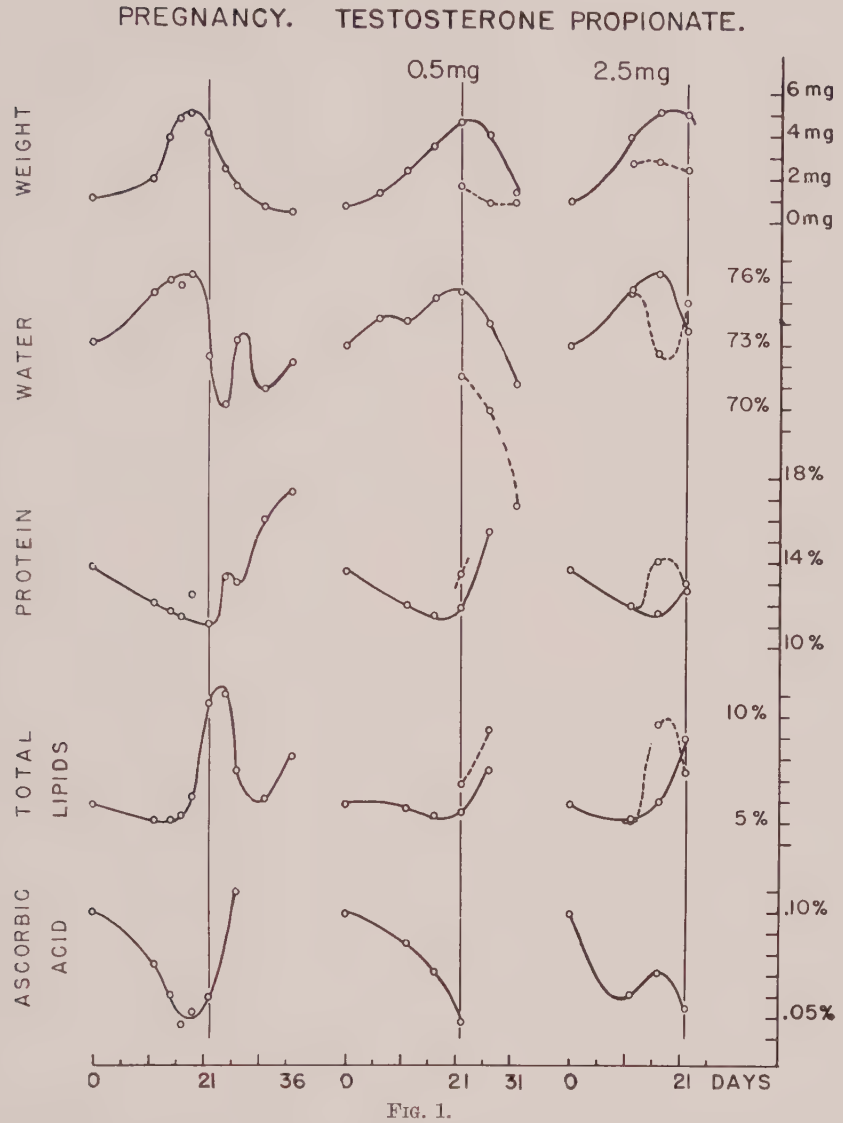

FIG. 1.

Weight curves and percentage values of water, protein, total lipids, and ascorbic acid of the corpus luteum in pregnancy and in experimentally induced hypertrophy.

corpora were transferred into weighing bottles to avoid drying of their surfaces. In the case of the controls, care was taken to dissect only the corpora of the last ovulation. These were easily distinguished from those of preceding ovulations by their greyish-white color and by a slight hyperemic surface protrusion marking the site of the stigma. Corpora lutea of previous ovulations were darker red and rounded. In the group of nursing rats, both sets of corpora lutea, those of pregnancy and those of lactation, were dissected out, weighed and analyzed separately. The data on corpora lutea of lactation are not reported here.

The moist corpora lutea were weighed in closed weighing bottles. The water content was determined by drying *in vacuo* over phosphoric anhydride to a constant weight which was usually reached within 18 hours.

The dried corpora were then extracted with ether in a micro-Soxhlet apparatus for 8 hours. The ether solution was transferred to a weigh-

ing bottle and the total lipids were weighed after evaporation of the ether. The lipids were then redissolved and aliquot parts used for colorimetric determinations by the Liebermann and the Zimmermann methods. The results were expressed as cholesterol and progesterone respectively by comparison with standard solutions of these substances. The non-steroid fraction was computed from the difference.

The total nitrogen content of the extracted corpora was then found by a semi-micro Kjeldahl method and this expressed as protein by the use of the factor 6.25.

Ascorbic acid was determined in the metaphosphoric acid extract of whole corpora lutea in separate specimens by titration with sodium 2.6 dichlorobenzenone-indophenol. Crystalline ascorbic acid was used as the standard.

The sum of the water, protein, and lipid expressed as percentages of the wet corpus luteum varied between 92.4 and 94.8, leaving between 5.2 and 7.6% of analyzed components such as carbohydrates and mineral salts.

Results and Discussion. The changes in the weight of the corpus luteum in mg and in the percentage of water, protein, lipids, and ascorbic acid calculated for wet tissue at various periods of the experiments are summarized in Fig. 1. The heavy vertical lines indicate the day of littering in the group of pregnant rats and the 21st day in testosterone treated rats respectively. The values obtained from animals which failed to respond in the usual way to the injected hormone are given in the curves with broken lines.

Before describing the findings, the values for day 0 require a brief consideration. They were obtained from our control animals killed during early diestrus, when the corpus luteum had become a rather solid anatomical structure. However, it should be pointed out that these corpora may not represent either the period of greatest corpus luteum function nor the earliest time of progesterone production in the normal animal. Recent experimental studies rather suggest that progesterone formation may occur even before ovulation.[14,17,18,19] It is for these reasons that we have not expressed the values in our experimental groups as percentages of the control values.

The 3 weight curves are essentially the same, indicating that the corpus luteum grows in the same fashion during periods of testosterone injections as it does during the course of pregnancy. There are but minor differences, in that the corpus luteum of pregnancy reaches the largest size at about the 18th day while the peak of the growth curve in rats injected with the small dosage is found at about the 21st day and that of rats receiving the larger dosage at about the 16th day. It seems reasonable to assume that there may be a dosage level between the two used here which may result in a growth curve fitting better yet that obtained during pregnancy. The dosage influences apparently the speed with which hypertrophy occurs. Whereas the results of diameter measurements suggested a plateaued weight between the 14th and 16th day of pregnancy and the day of littering, our curve indicates a maximum weight lasting only a short time and followed by a marked diminution in the size of the corpus luteum beginning several days before littering.

It is generally assumed that the enlargement of the corpus luteum is due to the increase in the size of the individual cells. No evidence has as yet been forthcoming which would indicate an increase in the number of specialized corpus luteum cells in pregnancy or after administration of steroid hormones. While an increase in cell size may be due to the deposition of unusual amounts of one or more substances, the analysis of the corpus luteum during its growth phase indicates that water, protein, lipids, and ascorbic acid increase simultaneously. This is readily seen when the actual weights of these various constituents are considered. If such weight curves are drawn they resemble that of the total weight of the corpus luteum. We have used the per cent values here in order to indicate the quan-

[17] Allen, E., Hisaw, F. L., and Gardner, W. U., 1939, Chapter VIII in *Sex and Internal Secretion*, page 458.

[18] Boling, J. L., and Blandau, R. J., *Endocrinology*, 1939, **25**, 359.

[19] Astwood, E. B., *Am. J. Physiol.*, 1939, **126**, 162.

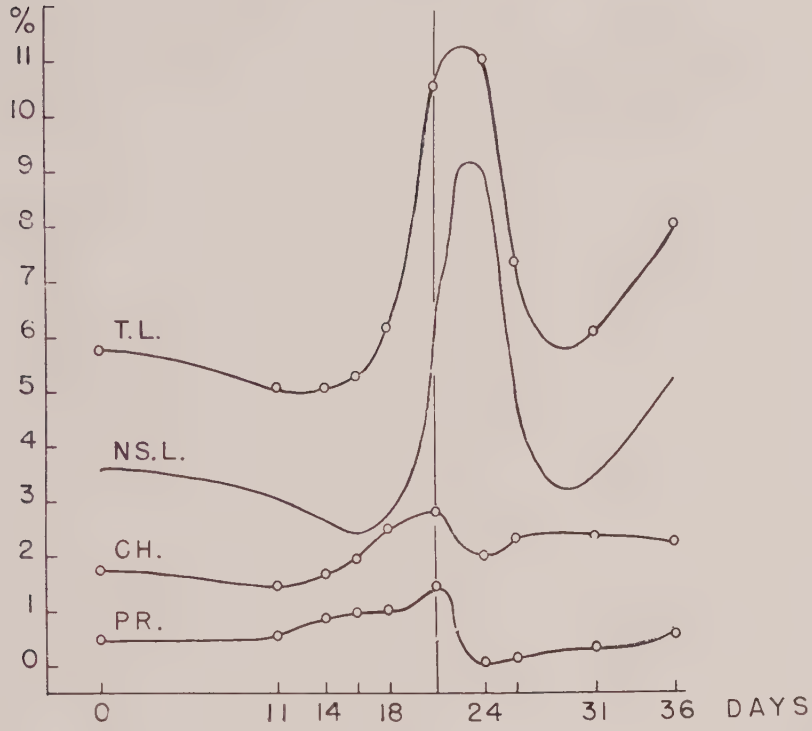

FIG. 2.
Percentage values for total lipids (T.L.), non-steroid lipids (NS.L), cholesterol (CH), and ketosteroids calculated as progesterone (PR) during the growth and regression of the corpus luteum of the pregnant rat.

titative relationship of the various cellular components to one another. It is seen then that during the period when the corpus luteum grows, water is present in larger proportions while protein, lipids, and ascorbic acid are found in relatively smaller amounts. Conversely, when the corpus luteum becomes smaller, the water content decreases both absolutely and relatively while the protein decreases absolutely although its concentration rises. These findings agree well with the general observation that growing tissues are richer in water than aged tissues.

The changes in the lipid fractions in relation to total lipids during the course of pregnancy and subsequent to parturition are given separately in Fig. 2. The total lipids show a slight decrease during the first 16 days which is followed by a marked rise evident at the 18th day of pregnancy. About 2 days following littering there is a drop in the total lipids which lasts but a few days, after which a second rise is observed. The decrease following parturition, though as yet unexplained, is of interest because it coincides with fluctuations in water and protein content. The possibility that the onset of lactation may have influenced the composition of the corpora lutea of pregnancy can only be suggested. Further analysis shows that the changes in total lipids are mainly due to those in the non-steroid fraction (glycerides and fatty acids), a finding which is in general agreement with the reports in the literature.[7,11,13]

The two lower curves in Fig. 2 give the values for cholesterol and ketosteroids, the latter calculated as progesterone. It is noteworthy that the ketosteroid fraction rises only slightly during the first half of pregnancy when progesterone action on the tissues is marked, while it increases sharply during the second half, reaching its highest concentration at about the day of littering. Taking into account the simultaneous growth of the cor-

pora lutea, the absolute amount of ketosteroids per corpus luteum can be calculated to have increased about 12-fold at that day. If this fraction represents principally progesterone, it may be possible that there is an increased release of this substance from the corpora lutea during the early periods of pregnancy. Such a mechanism is supported by the observations of Dempsey *et al.*, who found, using phenylhydrazine for histochemical demonstrations of ketosteroids, the smallest amounts during periods of greatest corpus luteum function. After littering a rapid lowering in ketosteroid concentration occurs, followed by a slow rise during the period of lactation.

During the first half of pregnancy the concentration of cholesterol actually decreases, but it rises during the second half of pregnancy, as does the ketosteroid fraction. It reaches its highest level at the day of littering, when it is increased sevenfold. Although the significance of cholesterol in the corpus luteum is unknown, the decrease in concentration may perhaps be connected with an increased permeability of the corpus luteum cell membrane or with the utilization of cholesterol in the production of progesterone.

The presence of ascorbic acid in the ovary was first emphasized in 1933[20] and attention was called to the corpus luteum as the tissue particularly rich in Vitamin C. It was further noted that the total amount of ascorbic acid varied with the functional state of the corpus luteum.[21–26] Although the importance of ascorbic acid and of its fluctuations with the physiologic states is unknown, it has been suggested that it is associated with the production of hormones in a non-specific way[27] and that an elevated amount suggests[24] or corresponds[15] to increased progestin secretion. When our data are expressed in absolute values, there is a rise and decline of the ascorbic acid values which corresponds to the general growth behavior of the corpus luteum. However, the ascorbic acid content, when expressed in relation to the weight of the corpus luteum, uniformly and significantly drops as the weight increases, and markedly rises as the weight falls. This indicates a dilution of extractable ascorbic acid rather than an increase in concentration. Because of the uncertainty as to the significance of ascorbic acid in corpus luteum function, the possibility still exists that there may be an increase in production of ascorbic acid which does not lead to an increased concentration in the corpus luteum because of increased utilization of ascorbic acid during the functional process. It should be remembered that the rat does not require exogenous ascorbic acid. This is in contrast to the species studied in the above mentioned articles and may possibly contribute to the differences noted.

As has been reported previously[16] deciduomata develop following traumatization of the uterus in rats injected with testosterone, provided functioning corpora lutea are induced. This test was used in the present experiment in order to supplement the analytical data with anatomical evidence of corpus luteum function. Of 42 rats injected with 0.5 mg daily for 11 and 16 days 37 animals, or 88%, developed deciduomata. This percentage was somewhat better than that obtained from all rats thus far subjected to the test, in which 24% of 137 rats failed to develop deciduomata. In the animals killed after 16 days of treatment, necrosis of the decidual cells was common, indicating that continued injections failed to maintain deciduomata beyond the usual 12 to 13 days.

Finally, the group of animals should be considered which responded differently to the injections than did the majority. These animals are represented in the curves with broken

[20] von Euler, H., *Ark. Kem., Mineral., Geol.*, 1933, **11**, 18.

[21] Huszák, St., *Z. f. Physiol. Chem.*, 1933, **219**, 275.

[22] Bessey, O. A., and King, C. G., *J. Biol. Chem.*, 1933, **103**, 687.

[23] Giroud, A., Leblond, C. P., and Giroux, M., *C. R. Acad. d. sc.*, 1934, **198**, 850.

[24] Biskind, G. R., and Glick, D., *J. Biol. Chem.*, 1936, **113**, 27.

[25] Policard, A. A., and Ferrand, M., *C. R. soc. Biol.*, 1936, **123**, 1081.

[26] Pincus, G., and Berkman, J., *Am. J. Physiol.*, 1937, **119**, 455.

[27] Bourne, G., *Australian J. Exp. Biol. and M. Sc.*, 1935, **13**, 113.

lines, and make up 39% of all the animals injected with testosterone. This reaction was observed more frequently during February and March than at any other time, the entire period of the experiments lasting from May, 1944, to April, 1945. The hormone preparation was the same before and after that period, so that variations in the testosterone can be excluded as the cause. Perhaps of still greater interest are the observations that these animals did actually show some degree of hypertrophy suggesting some form of response, and that they showed earlier the changes in composition which are typical for regression, such as loss in water and rise in protein and lipids. They formed a distinct group. No explanation can be offered as to the mechanism responsible for the lack of maximal response.

Summary. The changes in weight and in concentrations of water, protein, lipids, and ascorbic acid of the corpus luteum of the rat during pregnancy and during periods of injections with testosterone propionate at two dosage levels are reported. Comparison of the results permits the conclusion that the changes in weight and in chemical composition of the corpus luteum in experimentally induced hypertrophy are essentially the same as those occurring in untreated rats during the course of pregnancy.

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Comparison of the Cephalin-Cholesterol Flocculation with the Thymol Turbidity Test.

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The thymol turbidity test has been described recently by Maclagan[1] as an index of parenchymal liver dysfunction. Because of its simplicity the test gives promise of being of considerable aid in detecting disorders of the liver in which changes in the serum proteins are present. In clinical practice there is a marked parallelism between the thymol test and the cephalin flocculation test which also depends upon alterations of the serum pattern in certain liver diseases. Our studies indicate, despite this agreement, that the mechanisms of the two tests differ in important respects. In this paper some of these differences are described.

The thymol test is performed by adding 0.05 cc of the serum to be tested to 3 cc of saturated thymol in barbiturate buffered distilled water (pH 7.8). The intensity of the turbidity which develops within 10 minutes indicates the degree of active liver damage. Buffered distilled water without the addition of thymol does not give a positive test.

We have confirmed the findings of Maclagan that the test is usually positive in cirrhosis, hepatitis, etc., in which the cephalin flocculation is also positive. Notable exceptions have been observed, *e.g.*, the thymol test was positive and the flocculation negative in (1) certain lipemic sera such as in diabetes and in nephrosis, (2) biliary cirrhosis, (3) certain cases of metastatic neoplasm of the liver, (4) convalescent hepatitis, and (5) certain normal sera. The thymol test was negative and the flocculation positive in normal laboratory animals such as dogs and rabbits, and in certain rare cases of presumably normal individuals with no demonstrable liver disease.

Fundamental differences in the mechanism of the two tests can be demonstrated by studies on (1) effect of NaCl, (2) removal of lipids by

[1] Maclagan, N. F., *Nature*, 1944, **154**, 670; *Brit. J. Exp. Path.*, 1944, **25**; Hanger, F. M., *Tr. Assn. Am. Phys.*, 1938, **53**, 148; *J. Clin. Invest.*, 1939, **18**, 261.

ether extraction of frozen serum, (3) electrophoretically separated serum fractions, and (4) serum separated by ultracentrifugation.

1. *Effect of NaCl.* If the standard thymol solution is prepared with physiological salt solution instead of distilled water, all tests become negative. In contrast, the cephalin flocculation is routinely performed in salt solution which has no effect on the development of flocculation or precipitation.

The presence of salt in serum is probably accountable for the prozone noted in the thymol turbidity test. When a series of tubes containing reactive serum and a standard solution of thymol in distilled water were prepared (1/1, 1/2, 1/4, 1/16 . . . .) maximum turbidity was noted at 1/16. After removing the electrolytes from the serum by dialyzing against distilled water, turbidity was noted in the lower dilutions also.

2. *Effect of extraction of lipids from serum.* Sera from cases of hepatitis, malaria, and other disorders giving a positive thymol test were extracted by freezing in the presence of ether, as described by McFarlane,[2] a technic that has been shown to remove a large proportion of the serum lipids. In most instances the thymol test was made negative or weakly positive by ether extraction, while the cephalin flocculation test remained unaffected. Freezing alone had no effect on the reactions of the sera. Some exceptions were encountered in an occasional case of hepatitis, malaria, and disseminated lupus erythematosus where the thymol test remained positive after ether extraction.

The addition of whole normal serum, in equal amounts, to whole hepatitis serum, had no effect on the intensity of the thymol test, while the intensity of the cephalin flocculation reaction was strikingly reduced. On the other hand, the addition of whole normal serum to extracted hepatitis serum restored a negative thymol test to its original intensity. Extracted normal serum added to extracted hepatitis serum had no effect, and when extracted normal serum was added to unextracted hepatitis serum, the intensity of the thymol test was markedly reduced. Extracted normal serum could not be made positive by the addition of whole normal serum.

These findings tend to confirm the implication of Maclagan that lipids are essential to the thymol reaction. Further proof of this assumption was supplied by the finding that the addition of aqueous suspensions of cephalin, crude lecithin, or cholesterol, to extracted hepatitis sera restored a positive thymol reaction. Aqueous suspensions of the residues of the ether extracts from normal or hepatitis sera exhibited similar reactivating powers. On the other hand, there is no evidence that lipids play an essential part in a positive cephalin flocculation reaction, since there is no demonstrable effect on the intensity of the reaction after extracting or adding the lipids mentioned above.

3. *The thymol test on electrophoretically separated serum fractions.* Albumin and gamma globulin fractions from normal and abnormal sera were obtained by electrophoretic separation in phosphate buffer of pH 7.4. Negative thymol tests were observed with all albumin and gamma globulin fractions from both normal and abnormal sera. The addition of lipids and ether extract residues of serum to these fractions failed to produce a positive thymol test.

In contrast, gamma globulin fractions from normal and from hepatitis sera, as has been shown previously,[3] gave positive cephalin flocculation reactions. Albumin fractions from normal serum inhibited the flocculation of normal and hepatitis gamma globulins, while the albumin fraction from hepatitis serum failed to inhibit flocculation.

4. *The thymol test on serum fractions obtained by ultracentrifugation.* Whole hepatitis sera, extracted hepatitis sera, and extracted normal sera were centrifuged at 48,000 RPM for 3 hours. The sera were separated into three layers: (1) a clear supernatant, (2) a bilirubin stained middle layer, and (3) a small pellet. Flocculations and thymol test were set up with each layer. The supernatant layer from all 3 sera gave negative thymol and flocculation tests. The pellet gave positive flocculation and negative thymol reactions in all sera. The middle layer of whole hepatitis

[2] McFarlane, A. S., *Nature*, 1942, **149**, 439.

[3] Hanger, F. M., Moore, D. B., Pierson, P. S., and Moore, D. H., *J. Clin. Invest.*, 1945, **24**, 3, 292.

serum gave a positive thymol test, while the middle layer of normal serum and of extracted hepatitis serum gave negative thymol tests. The flocculation test was positive with the middle layer in all sera. The serum component giving a positive flocculation reaction appears to be present in both the middle layer and the pellet while that responsible for a positive thymol test is only in the middle layer, where the presence of lipid is also a requisite.

Discussion. Notable differences in the mechanisms of the cephalin flocculation and the thymol test are shown in the observations recorded above. The gamma globulin fraction, upon which the cephalin flocculation depends, is not an essential factor in the thymol test. Also the electrophoretically derived albumin fraction has no effect on the intensity of the thymol test. On the other hand the albumin fraction is an important factor in the flocculation reaction, since normal albumin inhibits flocculation while that derived from hepatitis serum fails to inhibit flocculation.

The presence of lipids is necessary for a positive thymol test, but has little effect on the cephalin flocculation reaction. There is no evidence that an abnormal lipid is present in hepatitis serum since the negative thymol test obtained after ether extraction can be made positive once more by a variety of lipid preparations and even by whole normal serum. Lipids alone give no reaction with the thymol reagent.

It must be assumed that an abnormal constituent in the serum in addition to lipids is required for a positive thymol test in cases of liver diseases. This assumption is based upon the observation that the addition of normal serum or of various lipid preparations restores a positive reaction only to extracted serum which was thymol positive before extraction. The disturbance lies apparently in the serum globulin fraction, but, as has been shown, does not involve primarily the gamma globulins.

Conclusions. The thymol turbidity test tends to parallel the cephalin flocculation test in most liver disorders and other disease, but notable discrepancies are frequent. Fundamental differences in the mechanisms of the two tests have been pointed out. Notable among these are, (1) the effect of salt on the 2 reactions, (2) the importance of lipids, (3) the role of electrophoretically derived albumin fractions and gamma globulins, and (4) the comparative reactions of serum fractions obtained by ultracentrifugation. It is recommended that both tests be employed for clinical purposes since different disturbances in the serum complex are detected by each.

We are indebted to Dr. Dan H. Moore of the Electrophoresis Laboratory, College of Physicians and Surgeons, Columbia University, for preparing the protein fractions used in these studies, and are most grateful to him for his help and interest during the progress of the work.

We acknowledge the valued assistance of Mr. Thomas Kantor for some of the studies on whole and extracted serum cited above.

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Blood Sugar Level and Autonomic Balance.*

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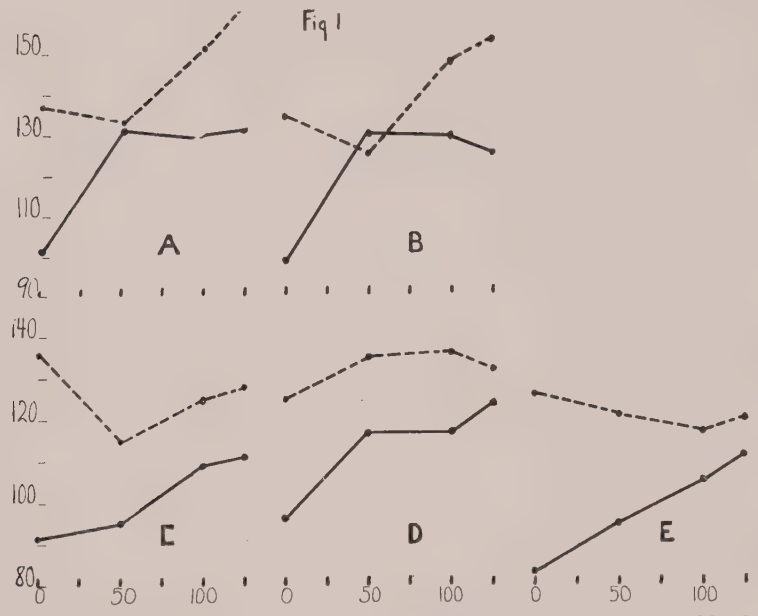
Previous investigations of Gellhorn and collaborators[1] have shown that the excitability of the sympathetic vasomotor center is increased with falling blood sugar level as indicated by the increased blood pressure response of man and animals to anoxia and to hyper-

* Aided by a grant from the Josiah Macy, Jr., Foundation.

1 Gellhorn, E., Ingraham, R. C., and Moldavsky, L., *J. Neurophysiol.*, 1938, **1**, 301; Ingraham, R. C., and Gellhorn, E., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 315; Kraines, S. H., and Gellhorn, E.,



Effect of 5 periods of 25 min. exposure to 280 mm Hg. on the blood sugar of normal rats. Ordinate—mg %; abscissa—time in min.
A, B—Rats of 21 days age.
C, D, E—Rats of 45 days age.
———————— rats fasted for 22 hr.
— — — — — — rats fasted for 4 hr.
Groups A, B, C, D, E consisted of 6, 5, 3, 5, and 6 rats respectively.

capnia. The effect was observed only when hypoglycemic states were compared with conditions in which the blood sugar was normal or above normal. The question as to whether lesser changes in blood sugar level not involving hypoglycemic states may alter the excitability of the sympathetico-adrenal (s.a.) system has not yet been investigated. Nor has its effect on the reactivity of the centers of the vago-insulin (v.i.) system been considered although Zunz and LaBarre[2] showed that cerebral hyperglycemia leads to increased secretion of insulin via the v.i. system. In the following experiments in which these centers were excited by exposure of the experimental animals (rats) to low barometric pressure an attempt was made to answer these questions.

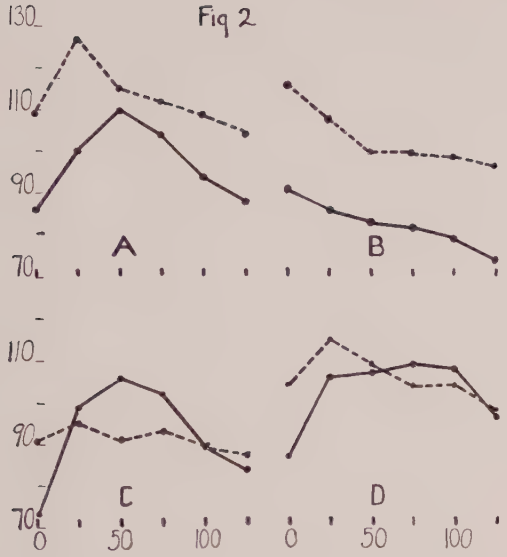
Since, as was shown previously, the autonomic balance is altered by age (Safford and Gellhorn[3]) it was thought of interest to study the influence of various blood sugar levels on this phenomenon in young and adult animals. Therefore, rats varying in age between 21 days and about one year were fasted for 4 and 22 hours and the influence of anoxia on the blood sugar level was determined. In view of the findings of Feldman, Cortell, and Gellhorn[4] this effect is the result of the action of anoxia on both s.a. and v.i. systems. In order to eliminate the effect on the s.a. system and to study the relation of the blood sugar level to the reactivity of the v.i. system alone experiments were performed on adrenodemedullated rats. Finally, a series of experiments was carried out on hypophysectomized animals. As a test for the activity of the s.a. and v.i. systems, the exposure of the rats to 5 periods (25 minutes each) of anoxia in the low pres-

Am. J. Psychiat., 1939, **95**, 1069; Gellhorn, E., Kiely, W. F., and Hamilton, S. L., *Am. J. Physiol.*, 1940, **130**, 256.

[2] Zunz, E., and LaBarre, J., *C. R. Soc. de Biol.*, 1927, **96**, 1400.

[3] Safford, H., and Gellhorn, E., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 98.

[4] Feldman, J., Cortell, R., and Gellhorn, E., *Am. J. Physiol.*, 1940, **131**, 281.



Effect of anoxia on adult rats. Procedure as in Fig. 1.

A—Normal rats.

B—Adreno-demedullated rats.

C and D—Hypophysectomized rats.

Rats in groups A and B were fasted 22 and 4 hours, but those of groups C and D 12 and 4 hours respectively.

Groups A, B, C, D consisted of 9, 8, 5, and 5 rats respectively.

sure chamber at 280 mm Hg[5] was chosen as in the preceding study.[3] The rats of the same age which were tested at 2 different fasting levels were litter mates.

Results. Fig. 1 (A and B), illustrating the results on 2 groups of young rats (21 days) shows, as previously reported, a marked s.a. response (hyperglycemia) as the result of exposure to low barometric pressure on animals fasted for 22 hours. If, however, the test was performed on their litter mates after a fasting time of 4 hours only, the results were quite different. Instead of showing a maximum in the hyperglycemic curve after exposure to low barometric pressure for 50 minutes as seen in the 22-hour fasted rats the blood sugar curve falls slightly at this time in the briefly fasted animals. In the later periods, however, the blood sugar rises also in these rats above the original level although, in general, to a lesser degree than was observed after a fasting period of 22 hours. These results are confirmed in the 3 groups of experiments presented in Fig. 1, C to E. The s.a. effect is invariably greater in the 22-hour fasting rats than is observed under otherwise similar conditions after the 4-hour fast. It is interesting to note that in 2 of the 3 groups of 45-day-old rats recorded in Fig. 1, C to E, a hyperglycemic effect is absent after the 4-hour fast although in the corresponding test after a 22-hour fast a distinct hyperglycemia occurred in all animals.

Similar experiments performed on adult rats (Fig. 2, A) shows likewise a greater and more persistent hyperglycemic action of the anoxia test on animals subjected to a 22-hour fast than is observed under similar conditions after a fast of 4 hours.

A final series on the effect of anoxia on rats at different fasting levels was performed on hypophysectomized rats in which the respective fasting times were 12 and 4 hours in order to obtain blood sugar levels comparable to those of the normal and adreno-demedullated groups. Since the responsiveness to anoxia as far as blood sugar is concerned gradually disappears in the first weeks after hypophysectomy[6] the experiments were performed only a few days after the operation. Fig. 2, C and D, shows first that the s.a. response resulting from anoxia is preserved in the hypophysectomized rats and second, that this response is markedly decreased at the higher blood sugar level.

Attention is called to the fact that in the second group (Fig. 2, D) both tests were completed within 5 days after the operation. After the 4-hour fast anoxia caused only a slight and brief rise in blood sugar whereas its effect after the 12-hour fast was much greater and more prolonged. Moreover, the effect of the 2 tests may be compared at the same interval after hypophysectomy since the 12-hour and 4-hour tests were performed on the fifth day in the first and second group respectively (Fig. 2, C and D). Here again a marked difference in the reactivity of the s.a. system is found which is determined by the initial blood sugar level.

[5] Van Middlesworth, L., Kline, R. F., and Britton, S. W., *Am. J. Physiol.*, 1944, **140**, 474.

[6] Unpublished observations.

The experiments presented thus far show clearly that in normal and hypophysectomized rats the blood sugar response to anoxia is modified by the prevailing blood sugar level. The higher the latter the less is the s.a. response. This phenomenon can be due either to an increased s.a. response or to a decreased v.i. response with falling blood sugar level or to a combination of both factors. Experiments on adreno-demedullated rats shown in Fig. 2, B, do not give any indication that the v.i. response is dependent on the level of the blood sugar. This experiment was repeated on another group of adreno-demedullated rats with similar results. It is therefore necessary to conclude from the experiments presented in this paper that in unanesthetized rats relatively slight changes in the blood sugar level resulting from different lengths of the fasting time significantly alter the reactivity of the autonomic blood sugar regulating centers. The balance existing between the v.i. and s.a. systems is altered in favor of the s.a. response with falling blood sugar level since no evidence could be obtained for a relation between the responsiveness of centers of the v.i. system to anoxia and the blood sugar level. The experiments show again the great stability of the centers of the v.i. system. Alterations in central autonomic balance induced by such diversified conditions as thyroxinization,[7] different age level,[3] and variations in the blood sugar concentration as the result of fasting seem to be due to the actions of these factors on the centers of the s.a. system only.

Summary. 1. The hyperglycemic effect of lowered barometric pressure (anoxia) on normal rats of different age groups is less in animals subjected to a 4-hour fast than in those fasted for 22 hours. 2. Similar results are obtained for hypophysectomized rats fasted for 4 and 12 hours respectively. 3. In contradistinction to the findings reported in 1 and 2, different durations of fasting have no effect on the vago-insulin system as shown by observations on adreno-demedullated animals.

It is concluded that the reactivity of the sympathetico-adrenal system increases with rising blood sugar level.

[7] Gellhorn, E., and Feldman, J., *Endocrinology*, 1941, **29**, 467.

15150

Dietary Requirements for Fertility and Lactation. XXXII. Rice and its By-products as Sources of Pyridoxine and Pantothenic Acid for Reproduction and Lactation.*

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While considerable information is now available on the distribution of various components of the vitamin B complex in natural foods and on their influence on growth, very little is known concerning their biological value as sources of the B vitamins for reproduction and lactation.

In the milling of wheat there is 86% loss of thiamine[1] and 83% loss of nicotinic acid but the losses in pantothenic acid and pyridoxine are only about 50%.[2] In the milling of rice there is 80% loss of thiamine[3] and 66% loss of nicotinic acid[4] and only about 50% losses

* Research paper No. 805, Journal Series, University of Arkansas. Published with the approval of the Director of the Arkansas Agricultural Experiment Station.

[1] Sherwood, R. C., Nordgreen, R., and Andrews, J. S., *Cereal Chemistry*, 1941, **18**, 811.

[2] Teply, L. J., Strong, F. M., and Elvehjem, C. A., *J. Nutr.*, 1942, **24**, 167.

[3] Kik, M. C., *Cereal Chemistry*, 1943, **20**, 103.

[4] Kik, M. C., and Van Landingham, F. B., *Cereal Chemistry*, 1944, **21**, 154.

TABLE I.
Rice and Its By-products as Sources of Pyridoxine for Reproduction and Lactation.*

Sources of pyridoxine	% of rice or its by-products in ration	Reproduction period (days)	No. of litters	Total young born	Young born dead	Young given to rear	Young reared	% young reared
Polished rice	15	56	4	25	2	21	0	0.0
Undermilled rice	15	57	3	22	0	17	0	0.0
Whole "	15	57	5	35	5	28	0	0.0
Polished "	30	100	8	68	3	47	14	29.8
Undermilled "	30	104	8	60	0	42	6	14.3
Whole "	30	96	5	42	4	29	6	20.7
Rice bran	5	128	11	90	0	66	18	27.3
" polishings	5	129	8	74	0	48	15	31.3
" bran	15	124	11	89	1	64	39	60.9
" polishings	15	134	10	103	0	60	41	68.3

* 5 females and 1 male in each experiment.

of pyridoxine and pantothenic acid.[5] The high content of the latter two vitamins in rice and its by-products stimulated an investigation on their biological value in this cereal and its by-products for reproduction and lactation.

The technic used for the lactation studies was essentially the same as that described in 1928.[6] The experiments were conducted in groups of 6 animals, consisting of 5 females and 1 male in each experimental set up. A total of 130 females and 26 males were used in this investigation. The animals were started on experiments when 24 to 31 days old and weighing from 40 to 55 g each. Each ration contained 22% purified casein (Smaco), 5% butter fat, 5% of Sure's salts No. 1,[7] and rice or its by-products at various planes of intake. The remainder of the ration consisted of cerelose (glucose). After the animals were 90 days of age and when the females weighed 155 to 160 g they were bred, at which time the ration was modified to contain 0.2% cystine which replaced an equivalent amount of cerelose, and the salt mixture was changed to Sure's salts No. 2, 5%.[8] The vitamin B complex supplements were given daily separately from the ration as follows: 20 μg thiamine, 20μg riboflavin, 20 μg pyridoxine, 6 mg choline chloride, and 200 μg calcium pantothenate. From the time of mating and through the periods of reproduction these were increased to 50 μg thiamine, 50 μg riboflavin, and 50 μg pyridoxine. During lactation they were further increased to 120 μg thiamine, 120 μg riboflavin, 120 μg pyridoxine, 9 mg choline chloride, and 600 μg calcium pantothenate. Those animals which received the rice or its by-products as a source of a specific B vitamin received no supplement of this particular vitamin. The results of previous work on quantitative requirements of the components of the vitamin B complex for lactation[9] served as a guide for dosage in this study. As a source of vitamins A and D, three drops of halibut liver oil were given once weekly to each animal, which was increased to 3 drops 3 times weekly per rat from the period of mating to the termination of the experiments. As a source of vitamin E, a concentrate was used, containing 34% tocopherols.† One drop of this concentrate, equivalent to about 8 mg of tocopherols, was administered once weekly to each animal. This was increased to 2 drops once weekly per animal from time of breeding and through the periods of reproduction and rearing of young.

The results of this investigation are summarized in Tables I, II, and III. From Table I it is apparent that, while polished rice, undermilled rice, and whole rice fed at a 15% plane of intake, as sources of pyridoxine, allowed reproduction to take place, lactation

5 Williams, V. R., Knox, Wm. C., and Fieger, E. A., *Cereal Chemistry*, 1943, **20**, 560.

6 Sure, B., *J. Biol. Chem.*, 1928, **76**, 673.

7 Sure, B., *J. Nutr.*, 1941, **22**, 499.

8 Sure, B., *J. Nutr.*, 1943, **26**, 275.

9 Sure, B., *J. Nutr.*, 1940, **19**, 57.

† Kindly furnished by Dr. P. L. Harris of the Distillation Products, Inc., Rochester, N.Y.

TABLE II.
Rice and Its By-products as Sources of Pantothenic Acid for Reproduction and Lactation.*

Sources of pantothenic acid	% of rice or its by-products in ration	Reproduction period (days)	No. of litters	Total young born	Young born dead	Young given to rear	Young reared	% young reared
Polished rice	15	41	2	15	0	12	0	0
Undermilled rice	15	40	3	29	2	18	0	0
Whole "	15	41	2	18	0	12	0	0
Polished "	30	83	8	67	4	45	0	0
Undermilled "	30	84	5	23	0	17	0	0
Whole "	30	83	5	41	1	29	0	0
Rice bran	5	66	5	38	0	29	0	0
" polishings	5	80	5	47	0	36	0	0
" bran	15	77	5	50	0	30	3	10
" polishings	15	70	5	46	0	30	0	0

* 5 females and 1 male in each experiment.

was an absolute failure. Increasing the rice to 30% in the ration permitted 14 to 30% success in rearing of young. As little, however, as 5% of rice bran or rice polishings in the ration resulted in about 30% success in lactation. Increasing these rice by-products to 15% in the ration resulted in marked improvement in rearing of young, *i.e.*, a lactation efficiency of 61 to 68%.

According to Williams, Knox, and Fieger[5] rice polishings contain 30.8 μg/g pyridoxine and 92.5 μg/g pantothenic acid and rice bran contains 32.1 μg/g pyridoxine and 71.3 μg/g pantothenic acid. Calculating on the basis of an average 20 g food intake during lactation, the ration containing 15% rice polishings furnished daily 277.5 μg pantothenic acid and only 92.4 μg pyridoxine. The 15% rice bran ration provided a daily intake of 213.9 μg pantothenic acid and 96.3 μg pyridoxine. Yet, such rations when fed as sources of pyridoxine allowed over 60% success in lactation, and when fed as sources of pantothenic acid produced 90 to 100% failure in rearing of young. The question arose: Is pantothenic acid unavailable in rice polishings and rice bran or are the pantothenic acid requirements for lactation greater than those of pyridoxine? That the latter was probably the case was suggested from the findings of Unna and associates[10] and from the report of Henderson[11] and coworkers that a daily dose of 80 to 100 μg calcium pantothenate is required for the optimum growth of the rat. That there is a relatively greater requirement of pantothenic acid than of pyridoxine for optimum growth of the albino rat is evident from the experiences of Unna and associates[10] who used 40 μg pyridoxine and 80 to 100 μg calcium pantothenate, and from the work of Pilgrim and Elvehjem[12] who used 20 μg pyridoxine and 200 μg calcium pantothenate. We used the latter two doses of these vitamins for growth. We used the 200 μg daily dose of calcium pantothenate, so that this same dose would insure a sufficiency for reproduction as well as for growth.

Since complete success in lactation in the rat has not yet been achieved with synthetic components of the vitamin B complex on purified diets fortified with optimum amounts of pure pantothenic acid, it is impossible at this time to compare the relative efficiency of this vitamin as provided by the rice polishings and rice bran rations with equivalent amounts of this pure vitamin. Accordingly, another series of experiments were conducted to throw further light on the problem. The results of these experiments are given in Table III. The supplementation of the 15% rice polishings ration with 300 μg calcium pantothenate daily furnished an additional amount of 253 μg of this vitamin, or a total of 277.5 + 253, or 530.5 μg. In other

[10] Unna, K., *J. Nutr.*, 1940, **20**, 565; Unna, K., Richards, G. V., and Sampson, W. L., *J. Nutr.*, 1941, **22**, 553; Unna, K., and Richards, G. V., *J. Nutr.*, 1942, **23**, 545.

[11] Henderson, L. M., McIntire, J. M., Waisman, H. A., and Elvehjem, C. A., *J. Nutr.*, 1942, **23**, 47.

[12] Pilgrim, F. J., and Elvehjem, C. A., *Arch. Biochem.*, 1945, **6**, 121.

TABLE III.
Influence of Supplementing Rice Polishings and Rice Bran with Calcium Pantothenate on Reproduction and Lactation.*

Sources of pantothenic acid	% in ration	Reproduction period (days)	No. of litters	Total young born	Young given to rear	Young reared	% young reared
Rice polishings	15	75 to 90	5	48	36	0	0.0
Rice polishings + 300 μg calcium pantothenate daily	"	80	7	67	42	21	50.0
Rice polishings + 600 μg calcium pantothenate daily	"	80	7	67	42	22	52.4
Rice bran	15	74 to 84	6	61	36	0	0.0
Rice bran + 300 μg calcium pantothenate daily	"	90	6	61	36	17	47.2
Rice bran + 600 μg calcium pantothenate daily	"	90	7	76	42	27	64.3

* 5 females and 1 male in each experiment.

words, it required about 7 times as much pantothenic acid to secure 50% success in lactation as for optimum growth and about 6 times as much pantothenic acid as pyridoxine (92.4 μg pyridoxine and 530.5 μg pantothenic acid) to secure relatively the same degree of success in lactation. Similar results were obtained with the 15% rice brand ration, but 300 μg additional calcium pantothenate did result in 17% increased success in rearing of young. The definite physiological requirements of the animal organism for pantothenic acid become more accentuated during lactation than during growth. Unna, Richards, and Sampson[10] found that the reduction to half the optimum dose of pantothenic acid during growth results only in less gain in body weight. These investigators found it necessary to reduce the daily dose of calcium pantothenate below 25 μg, in order to produce achromotrichia, and adrenal necrosis was produced in only 50% of the animals on a diet entirely deficient in pantothenic acid. However, even three to four times the dose of this vitamin required for optimum growth still results in 90 to 100% failure in lactation.

We know of no specific work dealing with relative quantitative requirements of pantothenic acid versus pyridoxine for optimum growth of the albino rat. Since, however, we have found in this laboratory 20 μg pyridoxine adequate for optimum growth and other workers[10,11] have reported 80 to 100 μg calcium pantothenate, equivalent to an average of about 75 μg pantothenic acid, it would appear that the optimum growth requirements of pantothenic acid are over three times those of pyridoxine. Our work then indicates that this ratio is much wider in lactation, *i.e.*, at least six times as much pantothenic acid as pyridoxine. This ratio cannot be considered as the final expression, since the optimum success in lactation we secured in this study was only 68%. The 32% infant mortality still to be accounted for may be due to some missing dietary factors or to imbalances of some essential constituents in the rations we employed. This work, however, establishes a greater requirement of pantothenic acid than of pyridoxine for the albino rat, which becomes more accentuated during the period of lactation.

Summary. Results are given on rice and its by-products as sources of pyridoxine and pantothenic acid for reproduction and lactation. The data in this study show that the requirements of pantothenic acid are much greater than those of pyridoxine for the albino rat, which become more accentuated during the period of lactation.

15151

Effects of Prolonged Irradiation of Mice with Fluorescent Light.*

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The carcinogenic property of ultraviolet irradiation is now well established. In fact, such radiant energy is one of the most effective of the many known carcinogens and the repeated exposure of mice[1-4] or rats[5] to ultraviolet rays of the region 2,800-3,341 Å[6,7] has been shown to result in a high incidence of skin cancer in several months. Furthermore, the production of neoplasms with ultraviolet light is not restricted to laboratory experimentation for a great majority of all the cancers of the skin in humans have also been attributed to prolonged exposures of excessive sunlight.[1,8] It therefore becomes apparent that all sources of ultraviolet irradiation to which man is exposed for long periods of time should be investigated for possible carcinogenic activity. One type of lamp that deserves such attention is the fluorescent type that has become so widely used in recent years. The light from this source approximates that of sunlight closely, and analysis of the emission spectrum of the daylight fluorescent lamp reveals that a very small amount of the wavelengths 3125 and 3341 Å are also present.[9] Although the intensity of the radiation of fluorescent lamps in the region below 3650 Å is negligible, it is possible that the total dose of these rays may approximate a carcinogenic amount in special cases where such lights are used repeatedly at short distances. The present report describes the effect of repeated and prolonged exposure of albino mice to the rays of a Westinghouse "Daylight" Mazda F fluorescent lamp.

Methods. Two groups of 48 strain C male albino mice 2 to 3 months old were housed in metal boxes on shavings and were fed a grain diet and water *ad libitum*. Six days each week they were put in screen cubicles[3] and placed under 2 parallel Westinghouse "Daylight" fluorescent lamps at a distance of 4 inches. They were irradiated for 6 hours a day the first 8 months and 4 hours a day the last 4 months for a total of 12 months irradiation. In order to facilitate the penetration of the light, the ears of the mice in one group were painted with purified mineral oil (U.S.P.) daily before exposure.[2]

The intensity of the light was measured with a thermopile, and the amount of energy emitted by the 3125 Å band was calculated from data obtained from a curve of the emission spectrum.[9] The intensity of the fluorescent lamp at the distance employed was 9.6×10^3 ergs/cm^2/sec., so that the mice were exposed to a total of 57.6×10^9 erg/cm^2 over the one year period. The mice were weighed and examined closely at regular intervals for any pathological changes.

Results. According to the emission spectrum, radiant energy ranging from 3000 Å to 10,500 Å is emitted by the "Daylight" fluorescent lamps,[9] and the 3125 Å band accounts for approximately 0.5% of the total intensity. Therefore, the amount of this wavelength received by the mice over a period of one year was 28×10^7 ergs/cm^2. This is slightly more than the minimum of 26.4×10^7

* This investigation was aided by a grant from the Jonathan Bowman Fund for Cancer Research.

1 Rusch, H. P., and Baumann, C. A., *Am. J. Cancer*, 1939, **35**, 55.

2 Rusch, H. P., Baumann, C. A., and Kline, B. E., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 508.

3 Rusch, H. P., Kline, B. E., and Baumann, C. A., *Arch. Path.*, 1941, **31**, 135.

4 Blum, H. F., Grady, H. G., and Kirby-Smith, J. S., *J. Nat. Cancer Inst.*, 1941, **2**, 259; 1942, **3**, 91.

5 Roffo, A. H., *Centralbl. f. allg. Path. u. path. Anat.*, 1935, **62**, 324.

6 Bain, J. A., and Rusch, H. P., *Cancer Res.*, 1943, **3**, 425.

7 Rusch, H. P., Kline, B. E., and Baumann, C. A., *Arch. Path.*, 1941, **31**, 135.

8 Blum, H. F., *J. Nat. Cancer Inst.*, 1940, **1**, 397.

9 Zimmerman, W. W., Report on Fluorescent Light, Lockheed Aircraft Corp., 1943, personal communication.

ergs/cm^2 reported to be carcinogenic when the mercury burner was the source of radiant energy.[3] The comparisons are not valid, however, since the calculations involving the mercury vapor lamp included all the wavelengths from 2900-3341 Å. Furthermore, the radiations from the mercury lamp were given over a short period and were considerably more intense than those received from the fluorescent light. This is important since the intensity must not fall below a certain minimum level if cancer is to be induced.[4]

Actually there is no direct evidence that the 3125 Å band will induce cancer. The carcinogenic portion of the spectrum has been shown to lie above 2500 Å and below 3341 Å as nearly as can be determined with the aid of special filters,[6,7] but with the methods now available it is not practical to measure the effects of individual bands within this range. However, since the 3125 Å band constitutes a fair portion of the total intensity between the region 2800-3341 Å emitted by the mercury vapor lamp, there is good reason to believe that it should be included in the carcinogenic range.

No tumors resulted from irradiation and no pathological changes of the eyes or other tissues were observed. The mice remained in relatively good health throughout the experiment and by 8 months 67% were still alive. During the last 4 months of the investigation, there was a slight weight loss and more deaths; but 32% of the mice were still alive when the experiment was terminated at the end of a year. The group of mice that received the applications of mineral oil to the ears were not essentially different from those not getting this treatment.

The results of the present experiment do not rule out the 3125 Å band as lacking carcinogenic activity but they do demonstrate that such activity does not exist with the doses applied. These negative findings are all the more significant because the thin skin of albino mice is especially susceptible to the carcinogenic effect of ultraviolet irradiation. In fact, tumors did not even occur on the ears of mice that had been painted with mineral oil just prior to raying, a procedure that accelerates the genesis of tumors by permitting a better penetration of the rays.[1] Thus it is safe to assume that the radiations at 3125 and 3341 Å as emitted by the fluorescent "Daylight" lamps have no carcinogenic effect on albino mice.

Summary. Two groups of 48 albino strain C mice were placed directly under the light of two Westinghouse "Daylight" fluorescent lamps for periods of 4 or 6 hours a day, 6 days a week, for one year. The ears of one of the groups were painted with mineral oil before raying to facilitate the penetration of the rays into the skin. Although the total amount of radiant energy applied over the total period was considerable, the intensity of ultraviolet radiations in the 3125 Å band was very low. There was no evidence of erythema, or neoplastic changes of the skin, or of irritation of the cornea.

15152

Comparison of the Golden Hamster to the Guinea Pig Following Inoculations of Virulent Tubercle Bacilli.

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This is a brief note on comparative studies of the reactions of the golden hamster (*Cricetus auratus*) and the guinea pig to inoculations with virulent human tubercle bacilli. The work was carried out for the following reasons: (1) The hamster has been used in the study of another Mycobacterial infection, *i.e.*, leprosy;[1] (2) It is reputedly susceptible to both the human and the bovine types of tubercle

[1] Burnet, E., *C. R. Acad. d. Sc.*, 1938, **207**, 690.

TABLE I.

A Quantitative Comparison (Expressed Numerically) of the Extensiveness of Disease Developing in Various Organs of the Hamster and Guinea Pig 120 Days After Individual Inoculations of 30,000 and 60,000 Virulent Human Type Tubercle Bacilli, H_{37} R_v.

Animals used	No. of animals in group	Dosage of micro-organisms	Average degree of tuberculosis				
			Lungs	Liver	Spleen	Lymph nodes	Total
Hamsters	10	30,000	0.7	1.0	0.9	1.5	4.1
Guinea pigs	10	30,000	2.5	2.6	3.3	3.4	11.8
Hamsters	10	60,000	0.9	0.9	1.1	1.9	4.8
Guinea pigs	10	60,000	3.0	3.0	4.0	4.0	14.0

bacilli;[2] (3) Its ability to thrive and breed in the laboratory environment[3] makes it a desirable animal for experimental and diagnostic work.

Twenty guinea pigs and 20 hamsters* were used. They were divided into two groups, each group consisting of 10 hamsters and 10 guinea pigs. Each animal in the first group was inoculated subcutaneously with 30,000 virulent tubercle bacilli of human type, $H_{37}R_v$. Each member of the second group received similarly 60,000 bacilli of the same strain. The number of bacilli was calculated from the actual dry weight of the inocula according to a technic previously described.[4] Twenty-four days after the infection each animal was skin tested with 0.1 cc of 5% and 10% Old Tuberculin. At the end of 48 hours, the guinea pigs showed areas of marked erythema, induration, and early necrosis. The skin of the hamsters exhibited no macroscopic reactions. At the end of 120 days the animals were sacrificed. The degree of macroscopic tuberculosis in each animal was evaluated by the method described by Steenken and Gardner.[5] The involvement of the spleen, liver, lungs, and lymph nodes was expressed in values proportionate to the extent and severity of the disease. The maximum rating of *4* in any organ was used to indicate widespread caseous disease. The maximum value of *16* for the animal as a whole signified advanced generalized tuberculosis.

The guinea pigs in both groups had extensive tuberculosis, but the hamsters showed relatively little disease. In the spleen of a few of the hamsters tubercle bacilli were demonstrated on smear. Microscopic sections of the lungs and spleen revealed only a few scattered small clusters of mononuclear cells.

Although others have reported that the hamster is susceptible to both the bovine and the human types of tubercle bacilli, their protocols do not bear out this impression. Balfour-Jones[2] infected 2 groups of hamsters intraperitoneally with 5 mg of "active" cultures of the human and the bovine types respectively. Two out of 3 hamsters in each group died in 5 weeks, and the third hamster showed marked evidence of infection when killed 8 weeks after inoculation. The author does not define his dosage of "5 mg" but if it was based upon wet weight the dose given to each animal was equivalent to *circum* 200,000,000 organisms;[4] if he used a dry weight basis the dose for each animal was approximately 1,500,000,000 microörganisms. For an animal to live 5-8 weeks after such a tremendous inoculum speaks more for its resistance than for its susceptibility. This relative resistance appears ever more striking when, as shown here, guinea pigs develop extensive generalized disease within 6 weeks after an inoculation of only 30,000 virulent tubercle bacilli. Apropos to the hamsters' relative resistance to the bovine and human types, is the further observation that they show no macroscopic lesions after being given as much as 50 mg of avian tubercle bacilli.[2] Furthermore, Gardner

2 Balfour-Jones, S. E. B., *Internat. J. Leprosy*, 1939, **7**, 77.

3 Laidlaw, Sir Patrick Playfair, *Internat. J. Leprosy*, 1939, **7**, 513.

* The hamsters were supplied to us through the courtesy of Merck & Company, Rahway, New Jersey.

4 Petroff, S. A., and Steenken, W., Jr., PROC. SOC. EXP. BIOL. AND MED., 1927, **24**, 958.

5 Steenken, W., Jr., and Gardner, L. U., *Yale J. Biol. and Med.*, 1943, **15**, 393.

and Delahant[6] have observed much less tuberculosis in hamsters than in guinea pigs after the inhalation of suspensions of tubercle bacilli (R_1 strain) in concentrations of 40-50 microörganisms per oil immersion field.

Conclusions. The hamster is highly resistant to doses of a virulent strain of the human type of tubercle bacilli that produces widespread tuberculosis in guinea pigs. Twenty-four days after infection 0.1 cc of 5% and 10% Old Tuberculin elicits no skin response. Therefore, in our opinion, we consider hamsters of little or no value in routine diagnostic procedures for tuberculosis.

[6] Gardner, L. U., and Delahant, A., personal communication.

15153

Relation of Plasma Level of Atabrine to Morphology and Motility of *Plasmodium vivax.**

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(Introduced by Carl Ten Broeck.)

The effects of therapeutic doses of atabrine on the appearance of malaria parasites in stained films were noted soon after this drug had come into use. They have been described in detail for *Plasmodium vivax,*[1] for *P. falciparum,*[2] and for *P. lophuræ,*[3] a parasite of birds. The early changes consist essentially of the appearance of pale-staining wisps of cytoplasm stretching out from the cell, aggregation of the pigment in a well defined clump, and fragmentation of the chromatin. In these studies no attempt was made to correlate the morphological changes with the concentration of atabrine in the blood and plasma. It was, however, found for the monkey parasite *P. knowlesi* that the maximal blood level of atabrine occurred within one-half hour after intramuscular injection of the drug, and that the parasite count began to diminish shortly after the peak atabrine level had been reached.[4]

In the present note it is desired to present observations made with living *P. vivax,* and especially to call attention to the possibly reversible effect of otherwise lethal concentrations of atabrine if they act over only a short period of time. The work constituted a small portion of an extensive study of relapsing *vivax* malaria in the Southwest Pacific theater of the war.

Cases of *vivax* malaria were selected which, on admission to the hospital, showed a parasite count of about 500 or more parasites per 500 white blood cells, with most of the parasites in the late ameboid stage. Before treatment was begun, a sample of blood was taken and used for the preparation of a wet film and for the preparation of plasma for atabrine assay. The patient was then injected intramuscularly with 200 mg of atabrine dihydrochloride in water. Blood samples were taken at intervals after the injection and used for wet films and for atabrine determinations. The wet films were all examined on a warm stage within a few minutes after preparation. Plasma atabrine levels were determined by the double extraction method of Brodie and Udenfriend.[5]

The results for 7 patients are presented in Table I. Before treatment it was easy to find in the wet films large motile parasites with

* This work was done in 1943 in Australia at the 92nd Evacuation Hospital and the 6th Army Training Center. It is a pleasure to acknowledge the whole-hearted cooperation of the staffs of both of these organizations.

[1] James, S. P., *Trans. Roy. Soc. Trop. Med. and Hyg.,* 1934, **28**, 3.

[2] Hühne, W., *Deut. trop. Z.,* 1942, **46**, 385.

[3] Hewitt, R. I., and Richardson, A. P., *J. Infect. Dis.,* 1943, **73**, 1.

[4] Chopra, R. N., Ganguly, S. K., and Roy, A. C., *Indian Med. Gaz.,* 1936, **71**, 443.

[5] Brodie, B. B., and Udenfriend, S., *J. Biol. Chem.,* 1943, **151**, 299.

TABLE I.
Relation Between Appearance of *P. vivax* Parasites and Plasma Level Following an Intramuscular Injection of Atabrine.

Patient	Time of sample	Motility of ameboid forms	Appearance of pigment	Parasites per 500 w.b.c.	Atabrine μg per l plasma
HK	1 hr before treatment	+ +	Normal. Fine, dispersed	1170	17
	15 min. after "	± —	Aggregated	1180	110
	75 " " "	— —	Dense clumps	1390	87
	195 " " "	± —	Normal in a few	970	27
Hn	15 " before "	+ +	Normal	330	17
	20 " after "	+ +	"	340	48
	80 " " "	— —	Clumped	170	80
	200 " " "	— —	"	200	52
Ml	30 " before "	+ +	Normal	510	15
	20 " after "	+ +	"	530	65
	170 " " "	— —	Clumped	380	43
By	30 " before "	+ +	Normal	660	0
	20 " after "	+ +	"	570	194
	60 " " "	+ —	Some normal, some clumped	840	69
	190 " " "	+ —	" " " "	700	39
Cy	1 hr before "	+ +	Normal	1690	5
	30 min after "	+ ±	Aggregated in a few	1960	187
	90 " " "	— —	Dense clumps	2160	182
	210 " " "	± —	Normal in a few	1690	50
In	15 " " "	+ +	Normal	2570	219
	75 " " "	+ —	Half normal, half clumped	2770	125
	10 hrs " "	+ +	Normal	2010	28
Bz	10 min " "	+ +	"	3530	182
	70 " " "	+ —	Half normal, half clumped	2580	100
	190 " " "	+ +	Mostly normal	2750	33

finely dispersed pigment. Some of the patients at this time showed a low concentration of atabrine in the plasma, since they had been taking the drug, but in doses too small to give effective suppression of the malaria. It will be noted that the plasma atabrine level rose rapidly, following the injection, to a peak, and then fell off rather rapidly. In each case, at the time of the peak atabrine level, or shortly thereafter, the proportion of normal motile parasites with finely dispersed pigment was greatly reduced, and instead there appeared non-motile parasites with a clumped pigment mass. In 4 out of the 7 patients, the samples taken more than 3 hours after the injection, when the atabrine level had fallen considerably, showed a greater proportion of normal appearing, motile parasites than the sample taken near the time of the peak atabrine level. Since no increase in parasite number occurred during the brief period of the observations, the results suggest that some of the parasites are only temporarily injured by a short exposure to an ordinarily more than effective concentration of atabrine, and that they recover when the atabrine concentration has dropped to a relatively low level. This would account for the need for repeated injections when malaria is treated parenterally with atabrine.

No change in the appearance or motility of the parasites was observed within the first 3 hours after the oral administration of quinine, although the concentrations of quinine were 6 to 10 mg per liter of plasma.

Summary. In patients infected with *P. vivax* and given a single intramuscular injection of atabrine, most of the large ameboid parasites were non-motile and showed clumped pigment at the time of the peak plasma level of atabrine, which occurred within an hour after the injection. Several hours later, when the plasma level of atabrine had fallen, there was some recovery of both motility and normal pigment distribution.

15154 P

Treatment of Sprue with Synthetic *L. casei* Factor (Folic Acid, Vitamin M).*

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The similarities of the sprue syndrome in man to the manifestations of vitamin M deficiency in the monkey[1,2,3] are obvious. It has been demonstrated that *L. casei* factor is curative for vitamin M deficiency in monkeys.[4] The availability of synthetic *L. casei* factor[5] has, therefore, prompted us to try this material in the treatment of 2 cases of non-tropical sprue which have recently been admitted to the Vanderbilt University Hospital. Striking clinical and hematological changes have occurred rapidly following the daily intramuscular injection of 15 mg of this synthetic material† as the sole therapeutic agent. Although remissions do occur in sprue, the results obtained indicate that folic acid may exert a specific effect in the treatment of the disease.

The first case treated was a 51-year-old white man who was admitted to the Medical Service of Vanderbilt University Hospital on September 24, 1945, with the complaint of sore mouth, diarrhea, weakness and weight loss over a period of 10 months. All criteria generally accepted for the diagnosis of non-tropical sprue were fulfilled. These were glossitis, diarrhea with increased fat in stools (37% of dried wt), marked loss of weight (38 lb), pigmentation of the skin, macrocytic anemia, moderate leucopenia, impairment of absorption as indicated by a flat oral glucose tolerance curve and vitamin A tolerance curve, characteristic gastrointestinal pattern on X-ray examination, and a very low serum carotene. The patient had free hydrochloric acid on gastric analysis. On October 4, 1945, he was started on intramuscular injections of 15 mg of the synthetic *L. casei* factor. Four days later the symptoms of glossitis had disappeared. On the 9th day of such therapy the reticulocytes reached a peak of 15.3% and a marked increase in thrombocytes was noted. The hematological improvement has continued (Table I), accompanied by marked general betterment including regeneration of the lingual papillæ, subsidence of the diarrhea and considerable gain in weight. Oral glucose tolerance and vitamin A tolerance curves became more nearly normal.

The second case is that of a 23-year-old white woman who was admitted to the Medical Service of this hospital on October 15, 1945, with the complaint of diarrhea, sore mouth, and general debility, which had its onset during the third trimester of pregnancy. These symptoms had persisted for 3 months until the time of admission, during which time the patient had lost some 34 pounds below her usual non-pregnant weight. Again, all of the usual criteria necessary for the diagnosis of sprue were fulfilled. Sternal aspiration revealed marrow typical of that seen in sprue

* A part of this study was financed through the Tennessee-Vanderbilt Nutrition Project by the Tennessee Department of Public Health, the Nutrition Foundation, Inc., and the International Health Division of The Rockefeller Foundation.

1 Langston, W. C., Darby, W. J., Shukers, C. F., and Day, Paul L., *J. Exp. Med.*, 1938, **68**, 923.

2 Day, Paul L., Langston, W. C., Darby, W. J., Wahlin, J. G., and Mims, V., *J. Exp. Med.*, 1940, **72**, 463.

3 Day, Paul L., *Vitamins and Hormones*, New York, 1944, **2**, 71.

4 Day, Paul L., Mims, V., Totter, J. R., Stokstad, E. L. R., Hutchings, B. L., and Sloane, N. H., *J. Biol. Chem.*, 1945, **157**, 423.

5 Angier, R. B., Boothe, J. H., Hutchings, B. L., Mowat, J. H., Semb, J., Stokstad, E. L. R., Subba-Row, Y., Waller, C. W., Cosulich, D. B., Fahrenbach, M. J., Hultquist, M. E., Kuh, E., Northey, E. H., Seeger, D. R., Sickels, J. P., and Smith, J. M., Jr., *Science*, 1945, **102**, 227.

† We are indebted to Dr. Stanton M. Hardy, Medical Director of Lederle Laboratories, for generous supplies of this crystalline synthetic "folic acid" used in these studies.

TABLE I.
Hematologic Response of a Case of Sprue Following Administration of Synthetic *L. casei* Factor.

Date	Therapy	Erythrocytes, millions per mm³	Hemoglobin, g per 100 cc	Reticulocytes, %	Leucocytes, thousands
9/24/45	None	1.35	6.5	2.9	4.32
10/ 1	"	1.56	6.0	—	5.75
4	15 mg *L. casei* factor	1.76	7.0	3.8	4.85
7	"	1.85	7.5	3.4	5.55
11	"	2.35	9.0	11.3	6.00
12	"	—	—	15.3	—
14	"	2.37	9.0	9.1	6.25
19	"	3.88	9.5	4.3	—

and pernicious anemia. Gastric analysis revealed free hydrochloric acid after histamine stimulation. Treatment with 15 mg daily of *L. casei* factor was instituted on October 19, 1945. On the fourth day of treatment reticulocytes had increased to 16.1% from a pretreatment level of 3.6% There was definite subjective improvement during this time. Her progress is being followed.

Castle[6] has reported that folic acid administered by mouth is without effect in doses up to 3.6 mg daily in patients with pernicious anemia, and Watson *et al.*[7] found *L. casei* factor ineffective in the treatment of 8 cases of "refractory anemia." There appear to have been published no previous experiences with this factor in the treatment of sprue.

Summary. The preliminary results here presented demonstrate that two cases of sprue have improved markedly following the parenteral administration of synthetic *L. casei* factor. It is reasonable to attribute the observed improvement to a specific effect of the substance administered, although one can not definitely rule out the possibility of spontaneous remissions in these patients.

[6] Castle, W. B., Ross, J. B., Davidson, C. S., Burchenal, J. H., Fox, H. J., and Ham, T. H., *Science*, 1944, **100**, 81.

[7] Watson, C. J., Sebrell, W. H., McKelvey, J. L., and Daft, F. S., *Am. J. Med. Sci.*, 1945, **210**, 463.

15155

Plasma Protein Concentrations in Adrenalectomized Rats Maintained on Sodium Chloride.*

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Adrenalectomy in the rat is followed by a decrease in serum albumin and an increase in serum globulin concentration.[1] Since adrenalectomized rats lose weight and reduce their food intake, it would appear that the serum protein changes are due to partial inanition in addition to mild cortical insufficiency. Furthermore, the adrenalectomized rat is in negative nitrogen balance.[2] When 1% sodium chloride is given to adrenalectomized rats as drinking water a body weight increase may be observed, more food is consumed and the negative nitrogen balance is improved.[2] The salt-maintained adrenalectomized rat is not normal in all respects and in this regard examination of the serum protein levels in a small group of rats revealed a subnormal serum albumin.[1] In the present investigation salt-

* Publication of the Bureau of Biological Research, Rutgers University.

[1] Levin, L., and Leathem, J. H., *Am. J. Physiol.*, 1942, **136**, 306.

[2] Rubin, M. I., and Krick, E. T., *J. Clin. Invest.*, 1936, **15**, 685.

TABLE I.
Adrenalectomy and Plasma Protein Concentrations.

Treatment	Body wt Start	Body wt End	Hemato-crit, %	Non-protein N mg/100 cc	Total protein g/100 cc	Albumin g/100 cc	Globulin g/ 100 cc	A/G ratio
(14) Adrenalx + NaCl	250	250	44.4±0.4*	78.0±4.0	5.97±0.09	3.36±0.09	2.61±0.12	1.29±0.08
(14) Normal—pair fed	247	246	46.1±1.1	72.0±3.6	5.97±0.11	3.46±0.17	2.51±0.10	1.38±0.11
(20) Normal—fed *ad. lib.*	235	252	45.4±0.7	54.0±0.8	6.37±0.07	3.86±0.09	2.51±0.10	1.54±0.10

*Mean deviation of the mean $= \epsilon = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$

maintained adrenalectomized rats are compared with pair fed and *ad libitum* fed normal rats to determine the effect on (1) body weight change, (2) food consumption, (3) plasma protein concentrations, and (4) organ weights.

Adult male rats of the Long-Evans strain were used at 140-150 days of age. Bilateral adrenalectomy was performed as a one-stage operation and the rats were placed in metabolism cages provided with 1% sodium chloride as drinking water. Two rats were kept in each cage and food intake was measured daily. Normal littermate controls given tap water were either pair fed or permitted *ad libitum* feeding. The experiment was terminated after 20-22 days at which time blood samples for plasma proteins were taken and each rat was autopsied. The manner in which blood samples were obtained as well as the methods of blood analysis used were the same as those reported previously.[3]

The average body weight of the adrenalectomized rats as well as of the pair fed normal rats was unchanged at the end of the 20-day experimental period. On the other hand, the *ad libitum* fed controls gained 17 g during this period (Table I). Food intake appeared to be the factor affecting body weight changes. The appetite of the salt-maintained adrenalectomized rats was subnormal. The effect of food intake on body weight was also reflected in those animals which lost weight. Eight of the adrenalectomized rats lost an average of 17 g despite the sodium chloride and the pair fed controls simulated the effect in an average loss of 13 g.

Food intake of the adrenalectomized rats receiving sodium chloride totaled 279 g per rat for the first 20 days following the operation. This was subnormal since *ad libitum* fed normals consumed an average 335 g during the same period. Although sodium chloride improves the appetite and consequently the food intake of the adrenalectomized rat our experiments and those of others[2,4] have shown that food intake is still below normal. On the other hand, Groat[5] found food intake to be normal in adrenalectomized rats on sodium chloride.

Examination of the plasma protein concentrations revealed that total protein was significantly lower in adrenalectomized rats on sodium chloride than in *ad libitum* fed control rats. This decrease was due entirely to a decrease in plasma albumin. A decrease in total plasma protein due entirely to a decrease in albumin concentration was also observed in normal rats pair fed with the adrenalectomized rats (Table I). Indeed, the plasma protein levels are a virtual duplication of each other, indicating that the food intake of the adrenalectomized rat on salt is sufficiently subnormal to alter the usual plasma protein levels significantly. Direct comparison of the protein concentrations in these experiments seems permissible since hemoconcentration was not observed in the adrenalectomized rat. Furthermore, the salt-maintained adrenalectomized rat is reported to have a normal plasma volume.[6]

Sodium chloride supplementation replaces, in part, adrenal cortical function and adrenalectomized rats on salt can manufacture plasma proteins as well as can normal rats,

[3] Leathem, J. H., *Endocrinology*, 1945, **36**, 98.

[4] Wyman, L. C., and tum-Suden, C., *Endocrinology*, 1945, **36**, 340.

[5] Groat, R. A., *Am. J. Physiol.*, 1941, **135**, 58.

[6] Hechter, O., *Endocrinology*, 1945, **36**, 77.

when both consume the same amount of dietary protein. Other adrenal cortical factors seem necessary for an over-all normal protein metabolism. This is further indicated by Winternitz[7] who found that the weight gain of adrenalectomized rats on sodium chloride was largely water and protein whereas pair fed normal rats exhibited an increase in fat but no change in protein. Our data would also suggest that digestibility is essentially normal in salt-maintained adrenalectomized rats although the low gastric secretion, with a low acid and enzyme content, of the adrenalectomized rat is not improved by salt.[8] Hartman *et al.*[9] have reported that the plasma proteins of the adrenalectomized dog are improved by sodium salts but not to the same degree as by cortical extract. Recent clinical studies on patients with Addison's disease reveal that these individuals have a low plasma albumin, in agreement with the results of animal experiments.[10]

The fresh weight of a number of organs was taken at autopsy to determine whether the adrenalectomy and/or the self-imposed starvation influenced organ weight. No significant organ weight differences for the pituitary, thyroid, testes, seminal vesicles, ventral prostate, spleen, kidney, or liver were obtained regardless of the groups compared.

Summary. The adrenalectomized rat maintained on sodium chloride for 20 days has a subnormal plasma albumin concentration. Pair fed rats exhibit the same low plasma protein concentrations and the same impaired growth rate. In the replacement in part of adrenal cortical function sodium salts appear to permit the adrenalectomized rat to manufacture plasma proteins in a normal manner although appetite is subnormal. Organ weights are not affected.

7 Winternitz, J. K., Ph.D. Thesis, Yale University, 1942.

8 Tuerkischer, E., and Wertheimer, E., *J. Endocrinology,* 1945, **4**, 143.

9 Hartman, F. A., Lewis, L. A., Thatcher, J. S., and Street, H. R., *Endocrinology,* 1942, **31**, 287.

10 McCullagh, E. P., and Lewis, L. A., *Am. J. Med. Sc.,* 1945, **210**, 81.

15156

The Sensory and Motor Axons of the Chorda Tympani.*

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The gross relations of the chorda tympani are well known and it is accepted that, functionally, it is a mixed motor-sensory nerve. The motor component is composed of preganglionic visceral efferent axons which pass to the submaxillary and associated terminal ganglia[1] and the sensory unit is made up of afferent axons which are distributed to the taste buds on the anterior two-thirds of the tongue[2,3] and possibly to the lingual mucosa.[2]

Until recently but little information has been available on the morphological types and sizes of axons in the chorda tympani. We have shown[4] that the nerve of the cat contains myelinated and unmyelinated axons. Both varieties of nerve fibers are of small caliber with the myelinated nerve fibers ranging from 1.5 to 6 micra in diameter while the unmyelinated axons are less than 1.5 micra

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1 Kuntz, A., *The Autonomic Nervous System,* Lea & Febiger, Philadelphia, 1934.

2 Cushing, H., *Johns Hopkins Hospital Bull.,* 1903, **14**, 71.

3 Lewis, D., and Dandy, W. E., *Arch. Surg.,* 1930, **21**, 249.

4 Foley, J. O., and DuBois, F. S., *J. Comp. Neur.,* 1943, **79**, 79.

across. On the average, furthermore, the myelinated preganglionic visceral motor axons are smaller, 1.5 to 2.5 micra, than the myelinated sensory nerve fibers. Finally some of the preganglionic motor and some of the sensory axons may be unmyelinated although relatively few of this anatomical type of nerve fiber is found in either functional group.

Few studies of a quantitative nature have been made on the chorda tympani.[5] We[4] have shown that 30% of the preganglionic visceral efferent axons and 45 to 55% of the afferent nerve fibers of the facial nerve of the cat enter the chorda tympani. Bruesch[5] was able to trace 48% of the myelinated afferent facial nerve fibers into the chorda tympani of the cat. Neither of these researches, however, give us pertinent quantitative information on the chorda tympani itself. Still wanting is more extensive data, from an adequate series of nerves, on the total number of nerve fibers, on the ratio of sensory to preganglionic visceral motor fibers and on the ratio of morphological types of axons in each functional category of the chorda tympani.

With the hope of filling these gaps in our knowledge of the anatomy of the nerve, quantitative studies have been made of the chordæ tympani from a series of cats and one dog in which the preganglionic visceral motor and sensory nerve fibers of the nerve have been segregated.

Methods. Six cats and one dog were subjected to one or the other of 2 surgical procedures. The preganglionic visceral motor axons were eliminated from the right chordæ tympani by severing them either in the rootlets of the facial nerve alongside the brain stem or by cutting them in the facial nerve within the pons.[4] These measures produced preparations in which only the sensory axons remained and in which, after appropriate technical steps, the number of sensory nerve fibers was enumerated. The approximate number of preganglionic visceral motor axons was then estimated by comparing the number of sensory axons which was found in the degenerated nerve with the number of combined motor and sensory nerve fibers which was counted in stained sections of the normal chorda tympani. The right superior cervical ganglion was removed in one cat and the right auricular branch of the vagus was cut in the roof of the tympanic bulla in another. This was done to check possible contributions of axons to the chorda tympani from either the cervical sympathetic or vagus since both of these nerves project axons into the facial nerve.[4] Following all types of surgery the animals were allowed to live for at least 14 days after which they were killed and the chordæ tympani were secured for neurohistological processing.

The nerves were stained in the block by one or the other of two variants[6,7] of the Ranson pyridine silver technic. After staining with silver the chordæ tympani were embedded in paraffin and cross sections of the nerves were made, toned and counterstained.[6] Toned and counterstained microscopic preparations, close to the origin of the chorda tympani from the facial nerve, were used for making differential counts of the myelinated and unmyelinated nerve fibers in the intact and degenerated chordæ tympani.

The strip method[8] was used for estimating the number of axons of all morphological types in the stained cross sections of the chordæ tympani. Estimates were made of the total number of nerve fibers and of the unmyelinated axons in a single cross section. The number of myelinated nerve fibers in the section was then arrived at by subtracting the number of unmyelinated axons from the total number of nerve fibers.

Results. There is wide variation in the number of combined sensory and motor axons in the intact chordæ tympani (Table I, unoperated side) of the cat. There may be as few as 1445 (cat 126) or as many as 2389 axons (cat 122) in these nerves. Within this combined sensory and motor group of axons the myelinated nerve fibers predominate. They range from 1231 (cat 126) to 1932 (cat 122) whereas the unmyelinated axons scale from 214 to 514. The chorda tympani of the dog (Table I) has more nerve fibers in it than does the chorda tympani of the cat. Approx-

[5] Bruesch, S. R., *J. Comp. Neur.*, 1944, **81**, 169.

[6] Foley, J. O., *Anat. Rec.*, 1938, **71**, 134.

[7] Foley, J. O., *Anat. Rec.*, 1939, **73**, 465.

[8] Davenport, H. A., and Barnes, J. F., *Stain Tech.*, 1935, **10**, 139.

TABLE I.
The Chorda Tympani.

Animal No.	Surgical procedure	Operated (right) side			Unoperated (left) side		
		Total axons	Myel. axons	Unmyel. axons	Total axons	Myel. axons	Unmyel. axons
Cat 131	Ganglionectomy Sup. cerv. ganglion	1755	1487	268	1861	1627	234
Cat 143	Section auricular nerve	1437	1231	205			
Cat 5	Intrapontine section roots	(1.46:1.00)* 1219s-833m	(1.45:1.00)* 1044s-719m	(1.53:1.00)* 175s-114m	2052	1763	289
Cat 2	Intrapontine section roots	(1.65:1.00) 1187s-720m	(3.00:1.00) 1054s-348m	(1.00:2.80) 133s-372m	1907	1402	505
Cat 126	Intracranial section roots	(2.39:1.00) 1019s-426m	(2.67:1.00) 896s-335m	(1.35:1.00) 123s-91m	1445	1231	214
Cat 122	Intracranial section roots	(1.00:1.10) 1135s-1254m	(1.00:1.15) 898s-1034m	(1.08:1.00) 237s-220m	2389	1932	457
Cat 123	Intracranial section roots	(1.98:1.00) 1335s-675m	(1.46:1.00) 888s-608m	(6.67:1.00) 447s-67m	2010	1496	514
Cat 121	Intracranial section roots	(1.18:1.00) 1046s-883m	(1.12:1.00) 888s-790m	(1.70:1.00) 158s-93m	1929	1678	251
Dog 1	Intrapontine section roots	(1.93:1.00) 2205s-1142m	(1.57:1.00) 1688s-1072m	(7.39:1.00) 517s-70m	3347	2760	587

*Ratio of sensory to motor axons.
s Sensory fibers.
m Motor fibers.

imately 1000 more axons and 800 more myelinated nerve fibers were enumerated in the intact nerve of dog 1 than in any cat within the cat series. There is no significant difference in the relative number of unmyelinated axons in the chordæ tympani of the two animals; those of the dog closely approximate the higher numbers found in the chordæ tympani of the cat group.

Section of the root fibers of the facial nerve of the cat and dog (Table I, operated right side) results in a diminution in the number of axons in the chorda tympani of both animals. The decrease is more marked for the myelinated than the unmyelinated axons. Section of the auricular nerve (cat 143), a sensory nerve, may reduce slightly the number of unmyelinated sensory axons in the chorda tympani of the cat. Removal of the superior cervical ganglion (cat 131) does not result in a diminution in either the myelinated or unmyelinated axons in the chorda tympani of the cat. The chorda tympani, therefore, near its origin from the facial nerve does not appear to contain motor axons from the cervical sympathetics. For all practical purposes, then, it is believed safe to consider as sensory the axons which remain in the chordæ tympani after the preganglionic motor axons of the facial nerve have been eliminated.

The sensory nerve fibers (Table I) in the chordæ tympani of the cat vary from 1019 to 1335. The single specimen from the dog contained 2205 sensory axons. Of these sensory axons the myelinated nerve fibers are in the majority; they range from 888 to 1044 in the chordæ tympani of the cat and are represented by 1688 axons in the chorda tympani of the dog.

There are usually fewer motor than sensory axons in the chordæ tympani of both the cat and dog. There may be as few as 426 or as many as 1254 preganglionic axons in the chordæ tympani of the cat (Table I). The chorda tympani of the dog held 1142 preganglionic axons. In the dog and in most of the cats the myelinated preganglionic axons of the chordæ tympani are more numerous than the unmyelinated motor axons; only occasionally does the unmyelinated variety predominate (cat 2).

The ratio of total sensory to total motor axons in the chordæ tympani of the cat varies (Table I) from near equality (cat 121) to a pronounced predominance of the sensory variety, 2.39 sensory to 1.00 motor axon (cat 123). In only one nerve (cat 122) is the ratio reversed; here, there is 1.10 motor axons

for each sensory nerve fiber. The ratio of myelinated sensory to myelinated motor axons closely parallels the ratio of total sensory to total motor axons, ranging from near equality (cats 121, 122) to as many as 3 sensory for each motor nerve fiber (cat 2). Although an approximate equality in the ratio of unmyelinated sensory to unmyelinated motor axons may occur (cat 122) the preponderance of the unmyelinated sensory (cat 123) or unmyelinated motor (cat 2) type may be much greater than that which occurs within the myelinated variety of nerve fibers.

An excess of sensory over motor axons is apparent in the chorda tympani of the dog. Of the total number of nerve fibers in the nerve there are almost 2 sensory axons for every motor nerve fiber. The ratio of myelinated sensory to myelinated motor axons is somewhat less, 1.57 sensory for each motor nerve fiber. There are many more unmyelinated sensory than unmyelinated motor nerve fibers in the chorda tympani of the dog, a ratio of 7.39 sensory for a single motor axon.

It is evident, therefore, that sensory axons predominate in the chorda tympani of the dog and in the majority of instances they are also the most numerous functional type of nerve fiber in the chorda tympani of the cat. Furthermore, this generalization applies not only to total axons but also to the myelinated and unmyelinated types of nerve fibers in the chorda tympani; for, there are also usually more sensory than motor nerve fibers in each of these morphological classes of axons.

Discussion. There is a dearth of data on the total number of axons in the chorda tympani. Most of the numerical studies on the chorda tympani have been made on sections in which only the medullary sheaths have been stained. In but few instances have quantitative analyses been done with microscopic sections which have been stained with silver. Foley and DuBois[4] and Schimert[9] studied the chorda tympani after staining it with silver. Schimert believes that the 1240 silver stained axons which he found in the facial nerve of one cat passed into the chorda tympani. Preliminary studies by Foley and DuBois on the silver stained axons of the facial nerve indicated that from 1300 to 2000 axons of the nervus intermedius entered the chorda tympani of the cat. The present study of individual chordæ tympani of the cat shows that actually there are from 1445 to 2389 axons in the intact chordæ tympani of the cat and 3347 axons in the normal chorda tympani of the dog. Schimert's figure of 1240 axons for the chorda tympani of the cat merits further inspection. It is believed that this figure should be revised downward since it includes all the axons of the nervus intermedius distal to the geniculate ganglion and it has been shown[4] that at least 12 to 15% of the sensory nerve fibers in the nervus intermedius of the cat, distal to the geniculate ganglion, do not enter the chorda tympani but pass, in the facial nerve outside the stylomastoid foramen, to the face.

Information on the total number of myelinated axons in the chorda tympani is scanty. Schimert[9] infers that 1349 axons with myelin sheaths passed into the chorda tympani of one cat. Here again he is unjustifiably taking for granted that all the myelinated axons in the nervus intermedius distal to the geniculate ganglion enter the chorda tympani. In this investigation 1231 to 1932 myelinated axons were enumerated in the normal chordæ tympani of 7 cats. Kure and Sano[10] report that the chordæ tympani of 2 dogs contained respectively 1642 and 1934 myelinated nerve fibers. In the present study 2760 myelinated axons were counted in the intact chorda tympani of one dog.

In a recent experimental study of the facial nerve Bruesch[5] has shown that 814 to 885 myelinated sensory nerve fibers remain in the chordæ tympani of the cat after its motor axons have been eliminated by sectioning them in the roots of the facial nerve. In the present research 888 to 1054 myelinated sensory nerve fibers were left in chordæ tympani which had been degenerated by surgical interruption of the motor axons of the facial nerve. The correlation between the figures as reported in this investigation and those of Bruesch are reasonably close. The chordæ

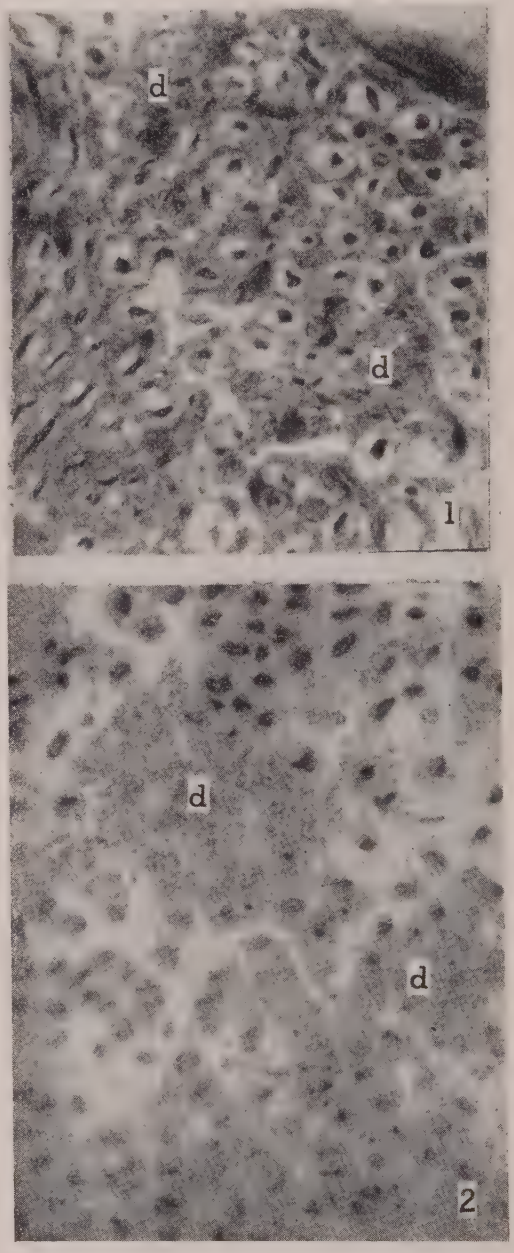
9 Schimert, J., *Z. f. mikr. anat. Forsch.*, 1936, **39**, 35.

10 Kure, K., and Sano, T., *Z. f. Zellforsch. u. mikr. Anat.*, 1935, **23**, 495.

tympani of 4 animals (Table I, cats 126, 122, 123, 121) contained from 888 to 896 myelinated sensory fibers. If the above values are compared with the number of sensory fibers (850 to 885) which Bruesch lists for the chordæ tympani of his cats B19, 23, 26, 27, Table 2, it will be seen that the results obtained, independently and in 2 different laboratories, are practically identical. The higher figures which were obtained by the writer in the chordæ tympani of cats 2 and 5 may be due to individual variation but they may also be a little higher because elimination of the motor fibers by intrapontine section may not be as complete as it is by destroying them in the rootlets outside the brain.[4]

Kure and Sano[10] contend that section of the roots of the facial nerve does not appreciably alter the number of myelinated nerve fibers in the chorda tympani of the dog. As a result they reason that the parasympathetic fibers of the nervus intermedius, which convey motor impulses over the chorda tympani, do not themselves enter the chorda tympani in significant numbers; most of them end on intercalary cells of the geniculate ganglion. Cells of the geniculate ganglion are believed by these investigators to give rise to the intrinsic parasympathetic fibers of the chorda tympani. The writer's findings do not support this conclusion, for not only are the axons of the chorda tympani of the dog significantly reduced in number after intrapontine section of the roots of the facial nerve (Table I) but actual areas of degeneration can be seen in silver-stained sections of the chordæ tympani of the dog (Fig. 1) and cat (Fig. 2) after the preganglionic parasympathetic motor axons have been deleted surgically central to the geniculate ganglion.

Since the sensory component is of recognized significance in conducting sensation, particularly taste, from the oral region, it is important to inquire if there are sufficient axons in the chorda tympani to supply the taste buds on the anterior two-thirds of the tongue. The right chordæ tympani of 6 cats (Table I) held an average of 1157 sensory axons. Hayes and Elliott[11] counted 575 taste buds in the fungiform papillæ of one side of the tongue of the cat. These separate findings



Photomicrographs of selected areas from silver-stained sections of the chordæ tympani of the dog and cat. Areas of degeneration are indicated by the letter d.

FIG. 1.

A field of the right chorda tympani of dog 1 showing two small regions of degeneration (d).

FIG. 2.

A field of the right chorda tympani of cat 122 showing two zones of degeneration (d).

[11] Hayes, E. R., and Elliott, R., *J. Comp. Neur.*, 1943, **76**, 227.

indicate that there are available at least 2 sensory axons in the chorda tympani for each taste bud on the anterior two-thirds of the tongue. In this investigation 2205 sensory fibers were enumerated in the right chorda tympani of the dog. Halliday[12] has demonstrated that the taste buds of the fungiform papillæ of the tongue of the dog vary from 1143 to 1774. Presumably there would be from 571 to 887 taste buds on one side of the dog's tongue in the territory supplied by the chorda tympani. In the dog it would appear that the ratio of sensory axons to taste buds is approximately 3 to 1. Since the sensory axons branch profusely as they approach the base of the taste bud[13] there would seem to be sufficient stem sensory fibers in the chordæ tympani of both the cat and dog for the supply of the sensory receptive cells of the taste buds on the anterior two-thirds of the tongue.

The observation by Bruesch[5] that myelinated sensory fibers from the auricular branch of the vagus enter the chorda tympani merits consideration. Bruesch showed that 26 to 37 myelinated axons of auricular origin were found in the chordæ tympani of cats after the motor and sensory fibers of the chordæ tympani had been deleted simultaneously by cutting the facial nerve distal to the geniculate ganglion. Bruesch's findings indicate that the number of myelinated sensory fibers listed in Table I should be reduced by 3 to 4% to arrive at the true number of myelinated sensory axons in the chordæ tympani which originate in the geniculate ganglion. It cannot but be wondered, however, if the variant introduced by the admixture of so few auricular axons would not be more than compensated for by the errors which are inherent in the quantitative method.[14] On the other hand, the possibility exists that unmyelinated axons may also enter the chorda tympani from the auricular nerve along with the myelinated nerve fibers since the auricular branch of the vagus is a predominantly unmyelinated nerve.[15] The intact chorda tympani of cat 126, Table I, is almost an exact replica of the surgical specimen from cat 143, there being a close parallel not only between the total number of nerve fibers but also between the myelinated and unmyelinated components of the 2 nerves. If these 2 nerves be compared it will be seen that there are a few less unmyelinated axons in the chorda tympani of cat 143 in which the auricular axons have been eliminated than there are in the intact nerve of cat 126. Whether this represents a true deficit or only a variation which might be expected in comparing nerves from 2 different animals cannot be answered. If the deficit does represent a diminution due to the loss of unmyelinated auricular fibers, it is of questionable significance, for the difference in the number of unmyelinated fibers in the 2 nerves (5%) would be more than counterbalanced by the error involved in staining and counting unmyelinated axons.[14]

Summary. The intact chorda tympani of the cat contains an average of 1955 combined sensory and motor axons; whereas, a total of 3347 sensory and motor nerve fibers is found in the normal chorda tympani of the dog (Table I).

An average of 1157 sensory axons remains in the chordæ tympani of the cat and 2205 sensory nerve fibers persist in the chorda tympani of the dog after the preganglionic motor axons are eliminated from the chorda tympani by cutting them in the facial nerve central to the geniculate ganglion.

It is estimated that the chorda tympani of the cat has, on the average, 798 motor nerve fibers in it. Approximately 1142 motor axons are present in the chorda tympani of the dog.

The majority of the motor and sensory axons in the chorda tympani of both the cat and dog belong to the myelinated variety of nerve fiber. In the cat an average of only 18% of the sensory nerve fibers and 20% of the motor axons is without myelin sheaths. In the chorda tympani of the dog 23% of the sensory axons was unmyelinated while just 6% of the motor axons was without myelin sheaths.

12 Halliday, J. C., *Ohio J. Science*, 1940, **40**, 337.

13 Kolmer, W., *Möllendorff's Handbuck der mikr. Anat. des Menschen*, Berlin, 1927, **3**, 171.

14 Foley, J. O., and DuBois, F. S., *J. Comp. Neur.*, 1937, **67**, 49.

15 DuBois, F. S., and Foley, J. O., *J. Comp. Neur.*, 1937, **67**, 69.

15157

Occurrence of Thiaminase in Marine Teleosts.*

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Diets containing whole raw carp have been found to be responsible for deaths due to B_1 avitaminosis in silver foxes.[1,2] Available thiamine was rendered inactive by the raw carp while still in the feed mixture.[3] This destruction of thiamine has been shown to be a split of the molecule into its pyrimidine and thiazole portions; the split is catalyzed by an enzyme, thiaminase, present in the viscera of raw carp and soluble in 10% salt solution.[4,5]

Deutsch and Hasler[6] examined a total of 31 species of fresh-water fish in the Great Lakes region and found the thiaminase to occur in about half of them. Examination by these authors of 9 marine teleosts, however, did not reveal thiaminase in any. They determined the presence of the enzyme in fish homogenates by their power to destroy the thiamine of added brewer's yeast. Thiamine analyses were made by the thiochrome method.

The only marine teleost which has been demonstrated to contain thiaminase is the Atlantic herring, *Clupea harengus*.[7] Both feeding tests and incubation with thiamine have given ample proof of the high thiaminase activity of this fish.[7,8,9] Green *et al.*,[10] on the basis of observations on the effect of diets fed at fur farms, suggest that Atlantic whiting, *Merluccius bilinearis*, has the power to destroy thiamine. On the other hand, according to the assays of Deutsch and Hasler[6] the whiting contains no thiaminase.

Large portions of the catch in certain fisheries are usually discarded,[11] although in recent years there has been a decided trend towards fuller utilization of this material. The possibility exists that such occasionally marketable or unmarketable (*i.e.*, "trash") species may at times be used for livestock feed on coastal farms. The presence of thiaminase in such species would preclude their use if raw and unprocessed. This paper reports the results of assays for thiaminase in 4 species of marine teleosts, namely:

Merluccius bilinearis; whiting
Prionotus carolinus; sea robin
Tautogolabrus adspersus; cunner
Tautoga onita; tautog or blackfish.

Thiaminase activity was determined by the method used by Sealock *et al.*[5] The viscera of fresh fish were minced in a Waring blendor with an equal volume of 10% saline in 0.1M phosphate buffer at pH 7.4. Large particles were spun down by centrifugation and 2 ml of the supernatant suspension (equivalent to about 1 g of fish) was transferred to a centrifuge tube together with 750 γ of thiamine hydrochloride and 1 ml of a 0.2M phosphate buffer at pH 7.4. The contents of each tube were adjusted to a volume of 5 ml with distilled water and then incubated for 2 hours at 37.5°. At the end of incubation 5 ml of 20% trichloracetic acid were added and the

* Contribution No. 000 from the Woods Hole Oceanographic Institution.

1 Green, R. G., Carlson, W. E., and Evans, C. A., *J. Nutrition*, 1941, **21**, 243.

2 *Ibid.*, *J. Nutrition*, 1942, **23**, 165.

3 Spitzer, E. H., Coombes, A. I., Elvehjem, C. A., and Wisnicky, W., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 376.

4 Krampitz, L. O., and Woolley, D. W., *J. Biol. Chem.*, 1944, **152**, 9.

5 Sealock, R. R., Livermore, A. H., and Evans, C. A., *J. Am. Chem. Soc.*, 1943, **65**, 935.

6 Deutsch, H. F., and Hasler, A. D., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 63.

7 Wolf, L. E., *Fisheries Research Bull. No. 2*, N.Y. State Cons. Dept., 1942, **2**, 16 pp.

8 Smith, D. C., and Proutt, L. M., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 1.

9 Melnick, D., Hochberg, M., and Oser, B. L., *J. Nutrition*, 1945, **30**, 81.

10 Green, R. G., Evans, C. A., Carlson, W. E., and Swale, F. S., *J. Am. Vet. Med. Assn.*, 1942, **100**, 394.

11 Merriman, D., and Warfel, H. E., *Trans. 9th N. Am. Wildlife Cons.*, 1944, 230.

protein allowed to flocculate with subsequent centrifugation. Standards received the same ingredients as the experimental tubes but the trichloracetic acid was added directly after the thiamine and incubation was omitted. Controls were run with the fish suspension subjected to 100° for 20 minutes prior to the addition of the thiamine followed by incubation. For assay two 4 ml samples of the resulting supernatant were used. A simplified modification[12] of the Melnick and Field[13] colorimetric diazonium method was employed to determine residual thiamine. It was found that the zeolite adsorption could be omitted without interference of color formation leading to erroneous analyses providing the absorption of light by the xylene extract was measured in a Klett-Duboscq visual colorimeter.

None of the 4 species of marine teleosts tested showed the presence of thiaminase in a saline suspension of its minced viscera. A similar suspension of whole minced quahogs (*Venus mercenaria*), which has already been shown to contain thiaminase,[9] destroyed 33% of the added thiamine in 2 hours. No appreciable heat-destruction of thiamine was found to occur under the conditions of the incubation.

The absence of thiaminase in most marine teleosts is still inexplicable. In the 13 species so far investigated only the herring, *Clupea harengus*, has been demonstrated to contain the enzyme. The conclusion of Deutsch and Hasler[6] that the whiting does not contain thiaminase has been confirmed. In their experiments with cod and haddock, Deutsch and Hasler[6] used eviscerated fish. Since the enzyme occurs primarily in the viscera, if at all, this leaves the status of these fish doubtful. Explanation of the occurrence of thiaminase in some fish and not in others may rest on a phylogenetic basis as has been suggested.[6] In that case extensive investigation of additional marine fish as well as freshwater, estuarine, and marine invertebrates and primitive chordates is requisite.

Summary. In 4 species of marine teleosts no thiaminase was found according to the procedure described. This brings to a total of 12 the marine teleosts in which there appears to be no thiaminase. In only one species, the Atlantic herring, has its presence been demonstrated.

I wish to thank Captain Jared Vincent for supplying me with the Atlantic whiting used in this investigation.

[12] Hochberg, M., and Melnick, D., *J. Biol. Chem.*, 1944, **156**, 53.

[13] Melnick, D., and Field, H., Jr., *J. Biol. Chem.*, 1939, **127**, 515.

15158

Utilization of Certain Rare Sugars by Microorganisms.

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The use of common sugars and alcohols in bacteriological work to differentiate and characterize species is well known. However, some of the so-called "rare" sugars and their derivatives have been used much less frequently for such purposes.[1] The failure to employ such substances in the past has been due in part to their high cost, or to the fact that they have been unavailable on the market.

In connection with other studies in the Division of Organic Chemistry at the State University of Iowa, several unusual sugars were prepared in sufficient quantities to permit their use in a few bacteriological studies. To

* We wish to acknowledge and thank Professor George H. Coleman for his help in the preparation of some of the sugars used in this study.

[1] See for example, Koser, S. A., and Saunders, F., *J. Bact.*, 1933, **26**, 475; Sternfeld, L., and Saunders, F., *J. Am. Chem. Soc.*, 1937, **59**, 2653.

TABLE I.
Utilization of Unusual Sugars in 24-72 Hr by Certain Microörganisms.

Organism	Turanose	Tagatose	Neolactose	Primeverose	Vicianose	β;β-Trehalose	4-(β-D-glucosyl)-D-manose	2-methyl-glucose	3-methyl-glucose	6-methyl-glucose	Levoglucosan	α-Schardinger Dextrin	β-Schardinger Dextrin
Esch. coli	AG	—	—	—	AG	AG	AG	—	—	—	—	—	—
Esch. coli	—	—	—	—	—	—	AG	—	—	—	—	—	—
Esch. coli -v.- communior	AG	AG	—	—	—	AG	AG	sl.A	—	—	—	—	—
A. acrogenes	—	A	—	—	—	—	—	—	—	—	—	—	—
A. oxytocum	AG	AG	—	AG	AG	AG	AG	—	—	—	—	—	—
K. pneumoniæ "995"	—	AG	—	—	—	AG	AG	—	—	—	—	—	—
K. pneumoniæ "1090"	—	AG	—	—	AG	AG	AG	—	—	—	—	—	—
K. pneumoniæ	—	—	—	—	AG	A	AG	—	—	—	—	—	—
Paracolon bacillus	—	—	—	—	—	AG	AG	—	—	—	—	—	—
E. typhosa "86"	—	A	—	—	—	—	—	—	—	—	—	—	—
E. typhosa "H"	—	A	—	—	—	—	—	—	—	—	—	—	—
E. typhosa "O"	—	sl.A	—	—	—	—	—	—	—	—	—	—	—
S. paratyphosa	—	AG	—	—	—	—	—	—	—	—	—	—	—
S. schotmuellerii	—	AG	—	—	—	—	—	—	—	—	—	—	—
S. schotmuellerii	—	AG	—	—	—	—	—	—	—	—	—	—	—
S. enteritidis	—	—	—	—	—	—	—	—	—	—	—	—	—
S. typhimurium	—	AG	—	—	—	—	—	—	—	—	—	—	—
Sh. dysenteriæ	—	—	—	—	—	—	—	—	—	—	—	—	—
Sh. paradysenteriæ "Flexner"	—	A	—	—	—	—	—	—	—	—	—	—	—
Sh. paradysenteriæ "Hiss Y"	—	—	—	—	—	—	—	—	—	—	—	—	—
Sh. paradysenteriæ "Sonne"	—	—	—	—	—	—	—	—	—	—	—	—	—
Sh. paradysenteriæ "Strong"	—	—	—	—	—	—	—	—	—	—	—	—	—
Bacillus macerans	AG	—	sl.A	AG	AG	AG	AG	—	—	—	—	AG	AG
" *polymyxa* (3 strains)	AG	—	A	AG	AG	AG	AG	—	—	—	—	AG	AG
Saccharomyces cerevisiæ	—	—	—	—	—	—	—	—	—	—	—	—	—
" *Carlesbergensis*	sl.A	—	—	—	—	—	—	—	—	—	—	—	—
" *Bayensis*	sl.A	—	—	—	—	—	—	—	—	—	—	—	—
" *globosus*	—	—	—	—	—	—	—	—	—	—	—	—	—

A—Acid; sl.A.—slight acid; AG—Acid and gas.

our knowledge these compounds have not been employed previously in this respect. Thus the present report is a brief communication dealing with the utilization of certain rare sugars and sugar derivatives by microörganisms.

Methods. Thirteen sugars were available for this study. Vicianose,[2] primeverose,[3] levoglucosan,[3] and β,β-trehalose[4] were all prepared by deacetylation of their acetates. Tagatose,[5] 2-methyl-D-glucose,[6] 3-methyl-D-glucose,[7] 6-methyl-D-glucose,[8] neolactose,[9] 4-(β-D-glucosyl)-D-mannose,[10] and turanose[11] were synthesized according to technics described in the literature. The α- and β-Schardinger dextrins were kindly furnished by Dr. T. J. Shoch of Corn Products Refining Co., Argo, Illinois. Concentrated solutions of the sugars were prepared separately in distilled water, and sterilized by passing through sintered glass filters. The concentrations were such that upon adding 0.1 ml of the sterile solution to 4.9 ml of previously sterilized meat-infusion broth (pH 7.2) a final concentration 0.2 or 0.5% sugar resulted.

Eighteen bacterial species and 4 yeasts were used in this work. The bacteria were stock strains, and the yeasts were obtained from Dr. L. J. Wickerham of the Northern Regional Laboratory in Peoria, Illinois.

The utilization of the sugars was determined by the conventional method of detecting acid and gas production; bromocresol purple was employed as the indicator of acid formation, and a Durham tube was used to detect gas. The bacteria were incubated at a temperature of 37°C, and the yeasts at 25° to 30°C. All tubes were observed for a period of one week.

Results. The results of this study are briefly summarized in Table I. It will be seen that the methylated sugars, levoglucosan, neolactose (galactose + altrose), and the Schardinger dextrins are not readily utilized by bacteria or yeasts. It is of interest that neolactose is not fermented by any of the coliform bacteria which normally utilize lactose. Primeverose and turanose were utilized by certain *Escherichia-Aerobacter-Klebesella* species or strains, and these sugars may be of some value for differential purposes, if a larger number of organisms were employed. Tagatose appears to have some importance in separating certain coli-aerogenes forms, and for differentiating between *Eberthella* and *Shigella* species. This sugar may also be of importance in characterizing some of the *Salmonella* species. Ordinary trehalose has an α,α configuration and is fermented by some of the coliform bacteria and *Salmonella* species.[12] The β,β-trehalose used in this study differs from the ordinary trehalose in that it is attacked by certain coliform bacilli, but is not utilized by any of the *Salmonella* species tested.

In general, none of the sugars is of any value in separating the species of yeasts employed: only *Saccharomyces Carlesbergensis* and *Sacch. Bayensis* produced slight acid from turanose.

Summary. Thirteen rare sugars, including turanose, tagatose, neolactose, primeverose, vicianose, β,β-trehalose, three methylated glucoses, and the Schardinger dextrins were tested for their utilization by 18 bacterial species and 4 yeasts. Some of these sugars appear to be of value for differentiating certain members of the enteric group of bacteria.

[2] McCloskey, C. M., and Coleman, G. H., *J. Am. Chem. Soc.*, 1943, **65**, 1778.

[3] McCloskey, C. M., Kirby, R., and Coleman, G. H., *Ind. Eng. Chem.*, 1944, **36**, 1040.

[4] McCloskey, C. M., Pyle, R. E., and Coleman, G. H., *J. Am. Chem. Soc.*, 1944, **66**, 349.

[5] Reichstein, T., and Bosshard, W., *Helv. Chim. Acta.*, 1934, **17**, 753.

[6] Lieser, T., and Leckzyck, E., *Ann.*, 1934, **511**, 137.

[7] Sundberg, R. L., McCloskey, C. M., Rees, D. E., and Coleman, G. H., *J. Am. Chem. Soc.*, 1945, **67**, 1080.

[8] Bell, D. J., *J. Am. Chem. Soc.*, 1936, **58**, 859.

[9] Richtmyer, N. K., and Hudson, C. S., *J. Am. Chem. Soc.*, 1935, **57**, 1716.

[10] Isbell, H. S., *J. Research Nat. Bur. Standards*, 1930, **5**, 1185.

[11] Hudson, C. S., and Pacsu, E., *J. Am. Chem. Soc.*, 1930, **52**, 2522.

[12] Bergey, D. H., Breed, R. S., Murray, E. G. D., and Hitchens, A. P., *Bergey's Manual of Determinative Bacteriology*, 5th Ed., Williams & Wilkins Co., Baltimore, 1939.

15159

Metabolism of Methyl and Benzyl Esters of Penicillin by Different Species.

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The various esters of penicillin are of potential pharmacological interest because of their fat solubility and because under some conditions they may be more stable than the ordinary salts. Mayer, Hobby, and Chaffee[1] first reported preparation of the methyl, ethyl, butyl, and benzohydryl esters, but the preparations used by them were so impure that interpretation of their data is difficult.

Cavillito *et al.*[2] used a preparation described as pure benzyl ester of penicillin G and concluded from studies carried out chiefly in mice that this preparation offers great promise of clinical usefulness. On the basis of metabolic studies in dogs we had previously abandoned work on the methyl ester of penicillin G[3] because it was not hydrolyzed in sufficient amounts to produce a therapeutic level of free penicillin in plasma or urine. Because of the discrepancies between our results and those of Cavillito *et al.* we undertook a comparative study of the metabolism of the methyl and benzyl esters of pure penicillin G by a variety of species of animals.

Methods. Dogs, mice, rats, monkeys, and rabbits were injected subcutaneously with commercial calcium penicillin, pure methyl ester of penicillin G, and the benzyl ester of pure penicillin G.* Samples of blood were removed by cardiac puncture at appropriate intervals of time and the concentration of free penicillin in the plasma determined by an agar cup test.† In the case of dogs complete urine samples were also collected and analyzed for the presence of penicillin. In dogs, monkeys, and rabbits, the same animals were used repeatedly for the study of the 3 preparations of penicillin. Because of the size of the other species it was necessary to sacrifice individual animals in order to obtain sufficient blood for analysis.

Calcium penicillin was administered as an aqueous solution; the methyl ester as a suspension in 2.5% aqueous starch emulsion unless otherwise stated. Because of the physical properties of the benzyl ester, it was administered as a peanut oil solution. Depending on the species, 3000 or 5000 International units of free or potentially free penicillin per kilogram were administered at each injection.

Results. Fig. 1 through 5 summarize the results obtained in the various species. The numbers in parentheses in the legend represent the number of animals used for determination of each point in the curve. Each point is a mean of the individual assays.

In all species administration of calcium penicillin resulted in significant plasma levels which reached a peak in 15-30 minutes, and fell off to zero in 2 hours. In general the larger the animal the higher was the peak plasma level for the same dosage per kilogram. Thus in the dogs which averaged 14.1 kilos,

1 Mayer, K., Hobby, G. L., and Chaffee, E., *Science*, 1943, **97**, 205.

2 Cavallito, C. J., Kirchner, F. K., Miller, L. C., Bailey, J. H., Klimek, J. W., Warner, W. F., Suter, C. M., and Tainter, M. T., *Science*, 1945, **102**L, 150.

3 Richardson, A. P., Ahlgren, M. W., and Miller, I., unpublished data.

* We are indebted to Dr. Elliott Shaw and Mr. Wm. Lott of the Division of Medicinal Chemistry for the sample of benzyl ester, and Dr. Max Adler and Dr. Oskar Wintersteiner of the Division of Organic Chemistry for the sample of the methyl ester. The methyl ester was a crystalline analytically pure preparation with sharp melting point. The benzyl ester was made from pure sodium penicillin G; it was a glassy, slightly colored solid, analyzing approximately for a benzyl ester of penicillin G.

† The biological tests for potency were carried out in the Division of Microbiology under the direction of Dr. Geoffrey Rake and Dr. Dorothy Hamre.

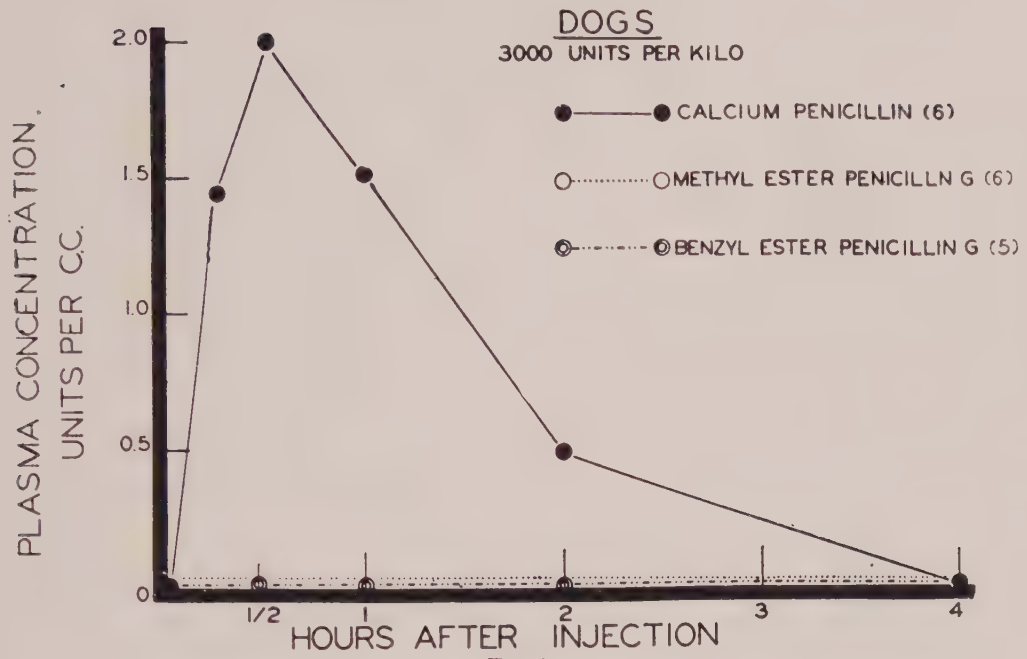

FIG. 1.

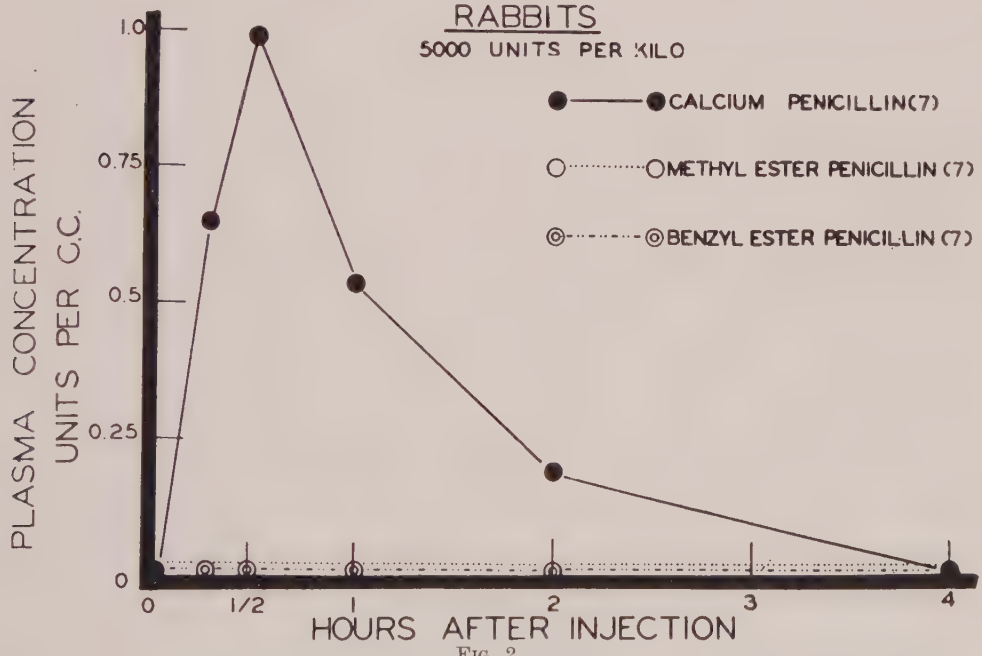

FIG. 2.

3000 units per kilogram gave an average plasma peak of 2.0 units per cc; whereas in mice which averaged 25.0 g, 5000 units per kilogram resulted in a peak level of only 1.1 units per cc.

In contrast to calcium penicillin, the results with the esters showed great differences depending on the species. In monkeys, rabbits, and dogs no free penicillin was detected in the blood at any time following administration.

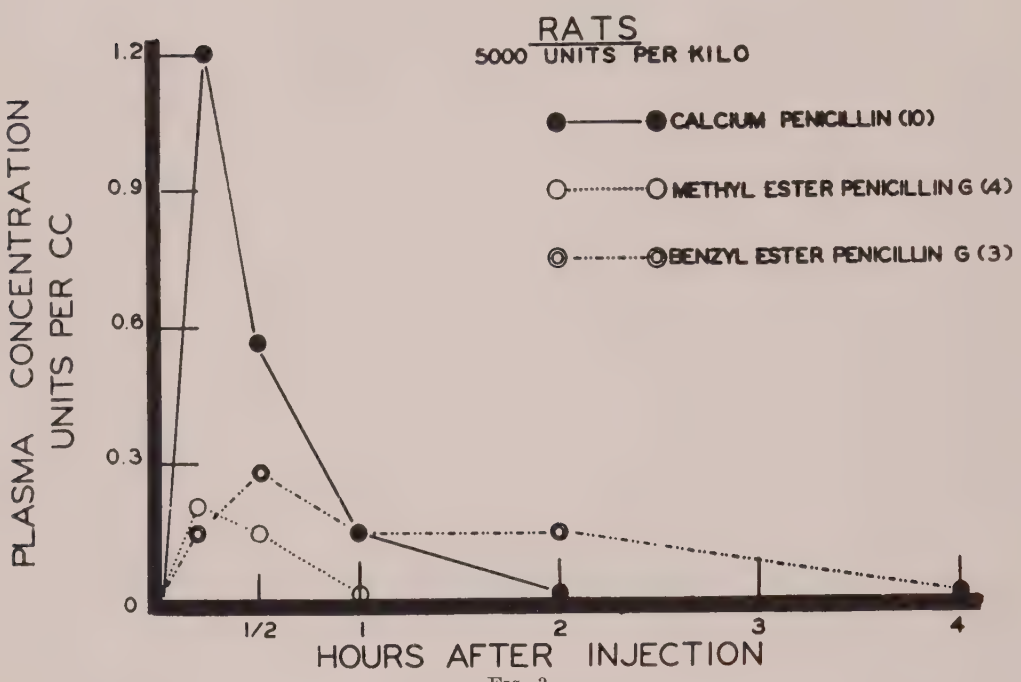

FIG. 3.

In mice, almost equivalent concentrations of free penicillin were observed following administration of the calcium salt and of the methyl ester. Plasma levels obtained with the benzyl ester in this species were somewhat lower, but since it was necessary to administer this preparation in peanut oil, it is likely that the delayed plasma peak noted was due to the vehicle. In confirmation of this conclusion the methyl ester was administered in peanut oil in one set of mice and as can be seen from Fig. 4, lower plasma levels were obtained and the peak of absorption was delayed.

The results obtained with rats were intermediate between those of mice and the other species. With both of the esters, significant levels of free penicillin were observeed in plasma, but not as high as in mice.

The only animals in which urine was collected were dogs. Up to 48 hours after the administration of the benzyl and methyl esters, less than 1% of free penicillin was recovered. This compares with 52% recovery when calcium penicillin was administered.[3]

Limited experiments were carried out in man with the methyl ester. Subcutaneous injection of 50,000 units suspended in starch failed to give a detectable blood level at any time up to 7 hours, and less than 0.5% free penicillin was recovered in the urine.

Discussion. Since neither of the esters used in this study had appreciable *in vitro* activity‡ it is reasonable to believe that any *in vivo* activity they may possess would be dependent on hydrolysis to free penicillin. It is apparent that there is great species difference with regard to hydrolysis of these esters. On the basis of very limited experience our studies indicate that man resembles monkeys, dogs, and rabbits in the manner in which they handle these two penicillin esters and it follows that it is questionable whether they will have any usefulness in man.

We have previously reported on the activity of the methyl ester in the treatment of relapsing fever in mice and found that it had the same activity as pure penicillin G.[4] On

‡ The methyl ester used has an approximate potency of 35 units per milligram, the benzyl ester 10 units per milligram, both determined by the cup test.

[4] Richardson, A. P., Walker, H. A., Loeb, P., and Miller, I., *J. Pharmacol.*, in press.

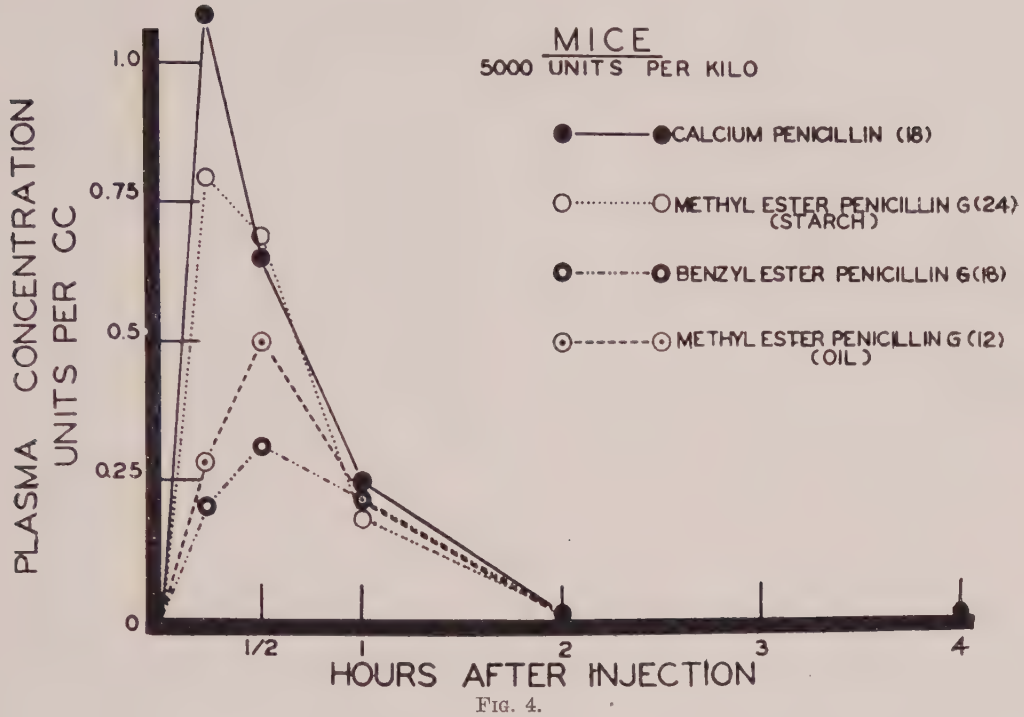

FIG. 4.

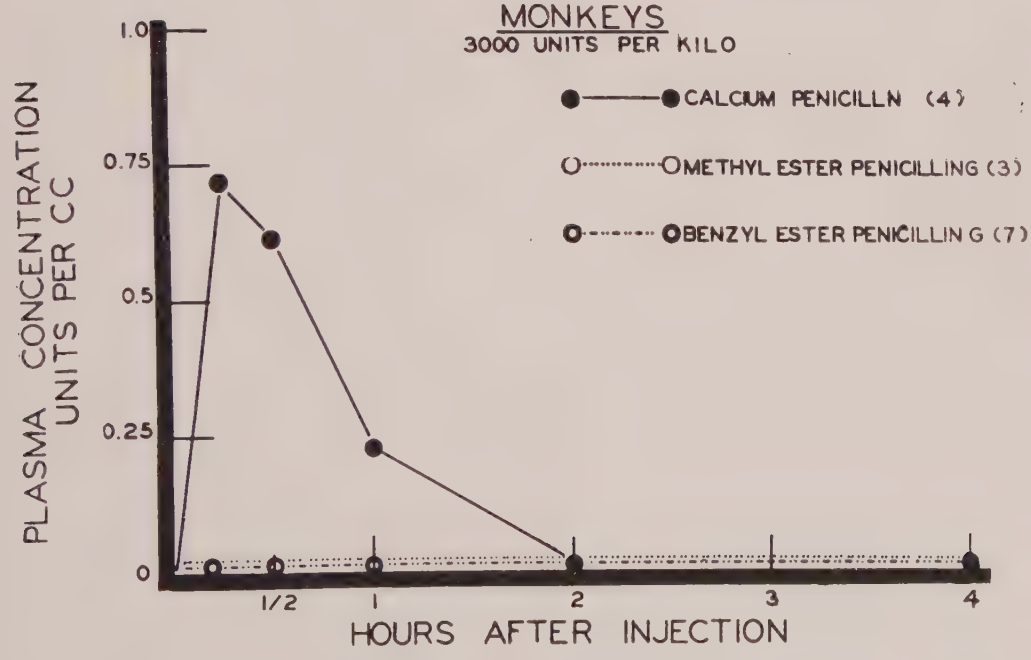

FIG. 5.

Fig. 1-5 represent plasma levels in various species following administration of calcium penicillin, methyl ester of penicillin G, and benzyl ester of penicillin G. Numbers in parentheses are number of animals used for determination of each point. Each point is a mean of all the determinations.

the basis of potentially free penicillin, Cavillito *et al.*[2] reported that the benzyl ester was more active than pure penicillin in the treatment of experimental streptococcal infections in mice. However, the benzyl ester was administered in sesame oil. Since this preparation is oil soluble and water insoluble, it is likely a more prolonged plasma level of free penicillin was obtained with the benzyl ester as compared to the sodium salt. The apparent increased activity of the benzyl ester in their experiments was therefore probably due to delayed absorption, rather than to any inherent increase in activity of the benzyl ester. The evidence available at the present time indicates that it would be hazardous to transfer results obtained in mice to man. Whether man can hydrolyze either of these esters to a significant degree can only be determined by further study.

Conclusions. Plasma levels following administration of benzyl and methyl esters of penicillin G have been determined in a variety of species of animals. Mice hydrolyze both of these esters rapidly and probably completely. Rats are able to hydrolyze the esters less completely. Monkeys, dogs, and rabbits show no appreciable plasma level of free penicillin following subcutaneous injection of ordinary doses of these esters.

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"Renal Hyperlipemia" in Dogs and Rats.*

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Previous work[1] has shown that unilateral and bilateral nephrectomy and also the administration of nephrotoxic agents like mercury bichloride, uranium nitrate, and potassium dichromate regularly increase blood lipid concentration in dogs. Hyperlipemia of the same pattern occurred, however, once after 4 sham operations, three times after 5 splenectomies, and was regularly observed after severe tissue necrosis. It was concluded that the kidneys of dogs, cats, rats, and monkeys are part of a mechanism which influences blood lipid concentration. These studies prompted further investigations to separate more clearly the influence of extrarenal tissue injury from the effect which renal tissue exerts on blood lipid concentration.

Methods and operative procedures have been described previously.[1] In order to eliminate the complicating factor of a general anesthesia, *unilateral nephrectomy* was performed *under local anesthesia* in 3 dogs.† The results are summarized in Table I and are identical with those obtained previously. A temporary hyperlipemia involving cholesterol, phospholipids, and total lipids was observed in all 3 animals. The highest values were obtained 4 to 8 days after operation with return to pre-operative levels after 10 to 14 days. It may be concluded that the general anesthesia used in former studies had no part in the development of the hyperlipemia following renal ablation.

When mercury bichloride was given by intramuscular or subcutaneous injection moderate tissue necrosis, resulting in ulcerations, was occasionally observed. It is conceivable that these lesions had an effect on the temporary hyperlipemia regularly observed after the administration of this nephrotoxic agent. *Mercury bichloride* therefore was *given by intravenous injection* in a 0.5% aqueous solu-

* Aided by a grant from the John and Mary R. Markle Foundation.

1 Heymann, Walter, and Clark, E. C., *Am. J. Dis. Child.*, 1945, **70**, 74.

† We are greatly indebted to Dr. Harry Goldblatt of the Institute of Pathology, School of Medicine, Western Reserve University, for performing the operations on the dogs.

TABLE I.
Effect of Unilateral Nephrectomy, Performed Under Local Anesthesia, on Serum Lipids of 3 Dogs.

Dog No.	Avg preoperative values in mg%			Highest postoperative value in mg%			No. of days after operation
	Cholesterol	Lecithin	Total lipids	Cholesterol	Lecithin	Total lipids	
43	120	260	400	342	550	1050	6
44	160	300	580	253	370	800	8
40	200	350	600	255	540	880	4

TABLE II.
Effect of Intravenous Injection of Mercury Bichloride on Serum Lipids of Dogs.

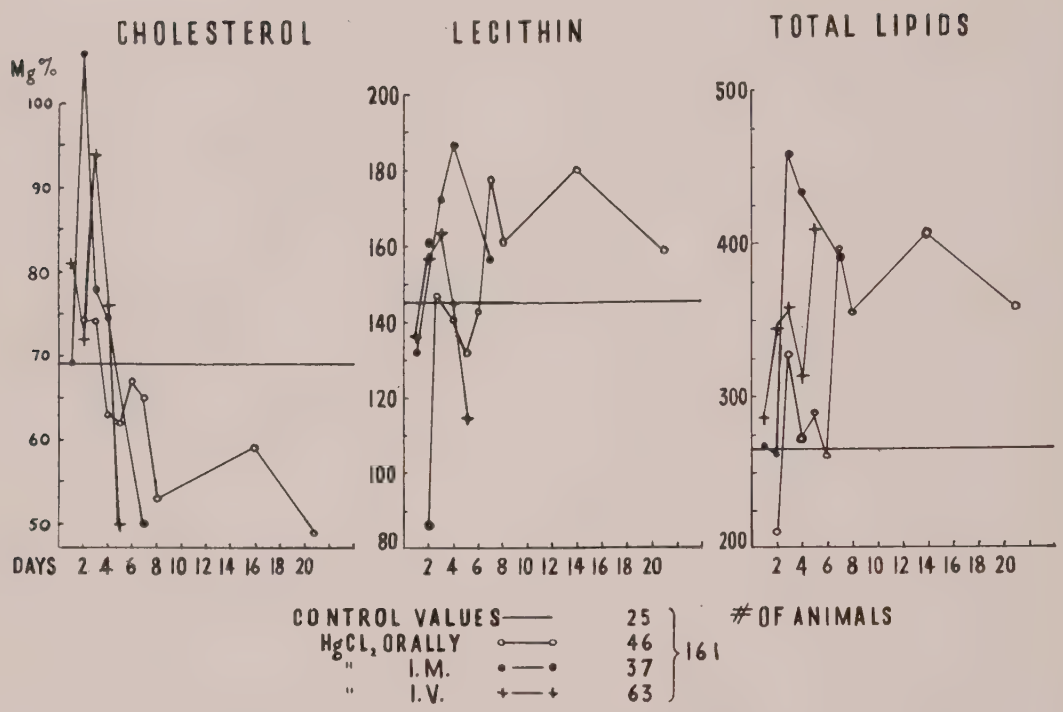
Dog No.	Dose, mg/kg	Avg values before injection, mg%			Highest value after inj. in mg%			No. days after inj.
		Cholesterol	Lecithin	Total lipids	Cholesterol	Lecithin	Total lipids	
49	2	98	280	490	342	460	1100	4*
44	1	100	350	620	275	520	980	4
57	1.5	188	400	680	519	710	1180	5*
46	3	105	275	480	219	578	1080	2*
47	1	124	240	610	200	410	910	3

* Died same day.

tion to 5 dogs (Table II), thus avoiding local injury at the site of injection. The two animals injected with 1 mg mercury bichloride per kg body weight developed a transient hyperlipemia identical in pattern and intensity with the reaction observed when 5 mg per kg body weight had been injected under the skin or into the muscles. The 3 remaining dogs injected with 1.5, 2, and 3 mg per kg died within 5, 4, and 2 days respectively and a continuous increase of cholesterol, lecithin, and total lipids was observed in their serum. It thus is shown that the hyperlipemia described in former studies was due to the action of mercury bichloride, and was not dependent on the resulting local tissue necrosis.

Effect of Mercury Bichloride on Serum Lipids of Rats. The effect of subcutaneous injection of mercury bichloride on serum lipids of 15 rats has been reported previously.[1] Increased values had been found, but more extensive studies had to be carried out. Fig. 1 shows average values obtained in 161 animals. When given by stomach tube from 6 to 30 mg of mercury bichloride was given per 1000 g of body weight. By intramuscular injection a dose of from 5 to 7 mg was used, and intravenously only from 1 to 4 mg per 1000 g of body weight was injected. It is seen that after intramuscular and intravenous injection of mercury bichloride, cholesterol, lecithin, and total lipid values rise and descend within the first 8 days. This immediate hyperlipemia is not observed when mercury bichloride is given orally. A delayed increase in lecithin and total lipids without corresponding rise of cholesterol values is observed, starting 7 to 8 days after its administration. These findings confirm the results obtained in dogs and it remains to be explained why in dogs and rats the oral administration of mercury bichloride is not followed by hyperlipemia, which is regularly observed when this agent is given by parenteral injection.

Effect of Carbon Tetrachloride on Serum Lipids of Dogs. Outside of renal ablation and tubular damage induced by nephrotoxic substances, severe tissue necrosis in dogs had been found to be followed by an identical hyperlipemia. One of the methods used to produce tissue necrosis had been the intramuscular injection of carbon tetrachloride which regularly led to the formation of severe abscesses. It had been concluded that not carbon tetrachloride, but the resulting necrosis of the tissues into which it had been injected was the cause for the increase in blood lipids. In order to test the validity of this conclusion, 15 cc of carbon tetrachloride was given by stomach tube to 2 dogs once, and 5 cc every 2 to 4 days to 4 dogs. The results are summarized in Table III. They show clearly that the oral administration of carbon tetrachloride decreased the concentration of cholesterol



BLOOD LIPIDS IN RATS AFTER ADMINISTRATION OF $HgCl_2$
(AVERAGE VALUES)

FIG. 1.

TABLE III.
Effect of Oral Administration of Carbon Tetrachloride on Serum Lipids of Dogs.

Dog No.	Avg values before administration of CCl_4 in mg%			Lowest value after administration of CCl_4 in mg%			No. days after first dose	cc	Dose
	Cholesterol	Lecithin	Total lipids	Cholesterol	Lecithin	Total lipids			
31	115	260	490	100	220	450	14	15	once
33	188	300	680	100	220	580	13	15	"
60	118	200	400	72	130	300	4	5	every other day
56	131	330	550	50	130	320	21	5	" 2-4 "
47	148	340	520	50	150	120	25	5	" 2-3 "
44	190	374	630	49	150	350	24	5	" 2 "

lecithin, and total lipids in serum. This strongly suggests that the hyperlipemia previously observed was not due to carbon tetrachloride but to the tissue necrosis resulting from its intramuscular injection. The hypolipemia observed after its oral administration may well be connected with the hepatic damage so produced.

Summary and Conclusions. (1) Unilateral nephrectomy was performed under local anesthesia in 3 dogs and was followed by a transient hyperlipemia involving cholesterol, lecithin, and total lipids. It thus is shown that the general anesthesia used in previous studies had no part in the development of the hyperlipemia following renal ablation.

(2) Mercury bichloride given by intravenous injection to 5 dogs produced in all animals a hyperlipemia identical in pattern and intensity with the reaction observed when intramuscular or subcutaneous injections had

been used. The local tissue necrosis which occasionally results from intramuscular and subcutaneous administration of mercury bichloride thus cannot be the cause of the hyperlipemia previously described.

(3) The same hyperlipemic reaction is observed in rats after intramuscular and intravenous injection of mercury bichloride. However, no immediate rise in serum lipids was observed after the oral administration of mercury bichloride. It remains to be investigated whether an effect of this agent upon the liver might explain the different action of enteral and parenteral administration of mercury bichloride.

(4) It was found in 6 dogs that the oral administration of carbon tetrachloride leads to hypolipemia involving cholesterol, phospholipids, and total lipids. This makes it clear that the hyperlipemia previously observed after it had been injected intramuscularly was not due to carbon tetrachloride but to the necrosis of the tissues in which it had been injected.

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Influence of Pyridoxine, Inositol, and Biotin on Susceptibility of Swiss Mice to Experimental Poliomyelitis.*

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Previous reports from this laboratory[1,2,3,4] have shown that the nutritional state of the Swiss mouse plays a role of varying importance in the susceptibility of this host to experimental poliomyelitis. Similar results with thiamine have been reported by the Pennsylvania group.[5] The most striking data were obtained with thiamine,[1] namely, mice fed a synthetic ration deficient only in this vitamin, exhibited a marked protection against infection with the poliomyelitic viruses, and indeed, in some series this resistance was 100%.[4] An attempt to ascertain the fundamental reason for these results[4] showed that the addition of pyruvate to thiamine optimum diets prolonged the incubation period of the infection in some instances, but did not manifest the marked resistance noted in frankly thiamine-deficient mice.

The specificity of the effect of nutrition in this disease is suggested by the fact that in pantothenic acid deficiency there is a definite increased resistance to Theiler's encephalomyelitis, but little or none to the Lansing strain of poliomyelitis virus,[2] while mice deficient in riboflavin manifest no altered susceptibility to Theiler's infection and a very slight increased resistance to the Lansing virus.[3] These results as well as those obtained with thiamine deficiency[1] suggest that factors such as inanition play a less significant role than the specific vitamins.

In the present report studies are presented for pyridoxine, biotin, and inositol deficiencies.

Methods and Materials. The synthetic optimal diets employed were composed of the following parts per 100: sucrose, 73; vitamin-free casein, 18; salt mixture, 4;[6] and corn oil, 5. The vitamin supplements per 100 g of diet

* These studies were aided by a grant from the National Foundation for Infantile Paralysis, Incorporated.

1 Rasmussen, A. F., Jr., Waisman, H. A., Elvehjem, C. A., and Clark, P. F., *J. Infect. Dis.*, 1944, **74**, 41.

2 Lichstein, H. C., Waisman, H. A., Elvehjem, C. A., and Clark, P. F., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 3.

3 Rasmussen, A. F., Jr., Waisman, H. A., and Lichstein, H. C., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 92.

4 Waisman, H. A., Lichstein, H. C., Elvehjem, C. A., and Clark, P. F., *Arch. Bioch.*, 1945, **8**, 203.

5 Foster, C., Jones, J. H., Henle, W., and Dorfman, F., *J. Exp. Med.*, 1944, **80**, 257.

6 Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, 1935, **109**, 657.

TABLE I.
Effect of Pyridoxine Deficiency on Susceptibility of Swiss Mice to Experimental Poliomyelitis.

Series No.	No. of days on diet before inoculation	No. of days after inoculation	% of mice paralyzed			
			Lansing (2%)			
			Deficient (21)*	Optimum† (21)		
28	35	5	19	10		
		6	29	24		
		7	39	24		
		9	52	57		
		10	57	62		
		13	81	71		
		15	81	76		
		28	86	81		
			Lansing (5%)		Theilers GDVII (1%)	
			Deficient (35)	Optimum (34)	Deficient (35)	Optimum (34)
40	27	6	3	15	0	0
		7	6	35	0	0
		8	14	44	0	0
		9	26	47	14	24
		10	31	47	29	35
		11	46	50	51	47
		14	66	62	63	79
		16	66	68	66	79
		18	69	68	71	82
		28	69	74	77	82
			Lansing (5%)			
			Deficient (25)	Optimum (27)		
44	33	6	16	26		
		7	24	37		
		8	28	44		
		9	48	52		
		10	52	56		
		12	56	67		
		18	68	70		
		28	68	70		

* No. of mice inoculated.

† This optimal diet contained 200 μg pyridoxine per 100 g diet, while in the other series, the optimum contained 300 μg.

unless otherwise stated were: thiamine, 0.3 mg; riboflavin, 0.3 mg; nicotinic acid, 0.5 mg; calcium pantothenate, 2 mg; pyridoxine, 0.3 mg; *i*-inositol, 100 mg; choline, 300 mg; para-aminobenzoic acid, 100 mg; and biotin, 5 μg. Through inadvertence in a previous paper[2] the thiamine content of the ration was not mentioned. Actually, 0.3 mg of thiamine was present in this ration as in all our diets save when otherwise stated. For the several deficiencies the respective vitamin was omitted from the ration. Each mouse received adequate amounts of oleum-percomorphum *per os* twice weekly.

Swiss mice bred in our own laboratory were used in all experiments, and split litter technic with consideration for sex and weight of mice was employed.

The viruses used, Lansing strain poliomyelitis and Theiler's encephalomyelitis (GDVII strain), were given by the intra-cerebral route using an inoculum of 0.03 ml and in the concentrations indicated. A comparison of the clinical picture obtained with our stock of GDVII with that reported by Olitsky[7] suggests that our virus is more nearly like their TO strain but of even greater virulence. However, until further verification is made the strain will be called GDVII. Uninoculated mice were maintained on both deficient and optimum diets to ascertain the effect of nutrition alone, and also to decide the optimum time for virus administration so as to obtain

7 Olitsky, P. K., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 77.

TABLE II.
Effect of Biotin Deficiency on Susceptibility of Swiss Mice to Experimental Poliomyelitis.

Series No.	Days on diet before inoculation	Days after inoculation	% of mice paralyzed					
			Theilers GDVII (10%)					
			Deficient (41)*		Optimum (42)			
41	48	8	17		2			
		9	22		12			
		10	46		33			
		12	78		62			
		14	83		74			
		20	88		83			
		28	88		83			
			Theilers GDVII (10%)					
			Deficient (28)		Regular opt. (33)			
46	40	8	21		6			
		9	29		33			
		10	32		39			
		11	50		55			
		12	54		64			
			Theilers GDVII (1%)					
			Deficient (31)	Opt. (28)	Regular opt. (29)			
48	30	7	13	0	0			
		8	19	4	10			
		9	26	28	28			
		10	32	54	59			
			Theilers GDVII (1%)			Lansing (5%)		
			Deficient (24)	Opt. (25)	Regular opt. (18)	Deficient (30)	Opt. (30)	Regular opt. (19)
50	34	8	4	0	11	13	13	5
		9	12	4	11	13	20	5
		10	21	12	22	17	29	16
		11	29	24	39	33	29	21
		12	29	24	56	33	29	21
		13	42	44	72	43	30	26
		14	50	56	89	47	33	32
		16	50	56	89	53	40	37
		19	50	56	89	53	43	53
		28	50	60	89	53	47	53

* No. of mice inoculated.

Optimal diets contained 100 μg of biotin per 100 g except in Series 41 where only 20-50 μg was present; in Series 41 the optimum diet contained 10% egg white and 10% casein, while in Series 48 and 50 the egg white was present as the sole source of protein in a concentration of 20%.

maximum paralysis at the peak of the deficiency.

In all experiments the animals were observed twice daily after inoculation for signs of flaccid paralysis; the experiments were terminated 28 days after administration of the virus.

Results. Pyridoxine. Three series involving 302 mice were studied using both Theiler's and Lansing viruses (Table I). One observes a lesser growth rate in mice deficient in this vitamin but no other gross manifestations of the dietary inadequacy are seen even after 6 to 8 weeks on the pyridoxine-free diet.

The end results in all series indicate no difference in susceptibility of the deficient or optimally fed mice to either virus. However, in Series 40 the results suggest an increased resistance to infection with Lansing virus on the part of the deficient mice during the early portion of the incubation period (5 to 10 days). Although there is a slight suggestion of

this same trend in Series 44 the differences are too small to be considered significant especially since the reverse phenomenon is seen in Series 28.

Biotin. Five series totalling 632 mice were studied with this deficiency. Four of these are listed in Table II, the fifth being omitted because of the low infectivity of the virus employed in that experiment.

For the production of biotin deficiency a diet containing powdered hen's egg albumin was employed. This was added to the diets in quantities sufficient to allow the avidin to combine with the biotin synthesized by intestinal microörganisms (usually 20%). In Series 41, egg albumin was present in the optimal diet as well as in the deficient ration, but in Series 46 the optimal diet contained no albumin. A change was made in the diets of Series 48 and 50 so that the egg albumin was present as the sole source of protein replacing the vitamin-free casein.

Biotin deficiency signs appeared in the mice after 4 to 8 weeks on the deficient ration. The first signs were a weight plateau, a roughening of the fur coat, a distinct dermatitis, and a halting high-stepping gait followed by the appearance of "spectacled eyes." Later the lips and nose became swollen and remained thickened, while incrustations of an exfoliative nature appeared over the entire body but more especially on the denuded areas. Similar findings have been reported by other investigators.[8,9] Curvature of the spine[10] appeared after 4 or 5 weeks and persisted throughout the deficiency. Control mice receiving adequate biotin remained in good health throughout the experiment. Mice showing extreme signs of biotin deficiency which were given as much as 50 to 100 μg of crystalline biotin per 100 g of diet exhibited gains in weight and improvement in the encrusted and denuded areas of the skin, but did not manifest complete return to normal despite 6 weeks of this therapy. Alleviation of the dermatitis upon the addition of biotin to the deficient diets was not as rapid in those mice receiving 20% egg white (sole source of protein) as in those animals receiving 10% each of egg white and casein.

The results of these experiments indicate no striking effect of biotin on the course of infection, but do suggest a slight but questionable tendency towards increased resistance of the optimally fed mice to Theiler's infection. In Series 50, a lowered incidence to Theiler's infection by both groups receiving egg albumin as compared with the optimal diet without egg white is seen, but the reason for this is obscure. The data of Series 46 and 48 are tabulated only up to 12 and 10 days respectively, since death of the mice due to an intestinal infection made the subsequent results impossible to correlate. The signs of this infection were an acute diarrhea, the anal region and tail becoming covered with fecal material which caused at times the complete closure of the anus. Cultivation of the stools of these mice on suitable media revealed large numbers of organisms which were identified as probable *Salmonella typhimurium,* and postmortem examination of the animals showed enlargement of the spleen and an acute inflammation of the gastro-intestinal tract. While this condition was observed in only 2 of the 5 biotin series, it suggests that mice deficient in this vitamin are more susceptible to naturally occurring salmonella infections than are animals maintained on a diet optimum in biotin.

Inositol. Both synthetic and natural diets were used to produce an inositol deficiency in 3 series of animals (478 mice). No marked signs of deficiency were found in animals fed the inositol-free synthetic diet save for a slightly soiled and roughened fur coat and an occasional area of alopecia. This is in contrast to the findings of Woolley[11] who noted marked alopecia in mice on a similar diet. In addition, this author has pointed out the importance of the level of pantothenate in the diet on the development of alopecia, but our attempts to produce denudation by varying the level of this vitamin were unsuccessful. The natural inositol deficient diet employed

[8] Nielsen, E., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 349.

[9] Emerson, G. A., and Keresztesy, J. C., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 358.

[10] Nielsen, E., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **144**, 405.

[11] Woolley, D. W., *J. Biol. Chem.*, 1941, **139**, 29.

TABLE III.
Effect of Inositol Deficiency on Susceptibility of Swiss Mice to Experimental Poliomyelitis.

Series No.	Days on diet before inoculation	Days after inoculation	% of mice paralyzed			
			Theilers GDVII (10%)		Lansing (5%)	
			Deficient (29)*	Optimum (31)	Deficient (35)	Optimum (34)
45	31	8	7	10	11	9
		9	14	26	14	15
		10	24	39	23	24
		11	52	55	26	24
		12	62	71	29	27
		14	86	90	34	30
		16	93	93	40	35
		18	97	93	51	38
		20	97	97	51	38
		28	97	97	51	41

Series No.	Days on diet before inoculation	Days after inoculation	Theilers GDVII (2%)		
			Deficient (25)	Opt., (25)	Synthetic opt. (17)
49	35	8	4	4	6
		9	20	8	18
		10	36	20	47
		11	56	48	76
		12	60	52	76
		14	72	68	94
		16	80	76	94
		17	84	84	94
		28	84	92	94

Series No.	Days on diet before inoculation	Days after inoculation	Theilers GDVII (10%)			
			Synthetic		Natural	
			deficient (38)	opt. (38)	deficient (39)	opt. (42)
51	44	8	0	5	8	7
		9	3	16	21	14
		10	18	24	26	26
		11	34	61	56	60
		12	47	79	74	71
		13	61	79	82	79
		14	63	84	90	81
		16	68	89	95	83
		17	71	89	95	83
		20	74	89	95	86
		28	74	91	95	86

* No of mice inoculated.
Series 45: synthetic ration.
Series 49: natural ration.

was the one suggested by Cunha *et al.*[12] which consisted of ground yellow corn 75.35, soybean oil meal 17.50, alfalfa meal 5.00, $CaHPO_4$ 1.00, NaCl (iodized) 0.50, $MnSO_4$ 0.01, haliver oil 2 drops weekly, a "folic acid" concentrate equivalent to 2 g liver extract[13] and 300 μg pyridoxine. While rats show alopecia on such a regimen, mice fed this diet did not develop any loss of hair throughout the experimental period. However, the use of this diet presented an opportunity to compare the effects of synthetic and natural foodstuffs on the course of virus multiplication.

With this deficiency as in the 2 previous ones there was no consistent difference between deficient or optimally fed mice to infection with either virus. Only in Series 51 was there a consistent lowered incidence of paralysis exhibited by those mice fed a synthetic ration deficient in inositol. The reasons for the increased resistance of both deficient and

12 Cunha, T. J., Kirkwood, S., Phillips, P. H., and Bohstedt, G., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 236.

13 Hutchings, B. L., Bohonos, N., and Peterson, W. H., *J. Biol. Chem.*, 1941, **141**, 521.

optimally fed mice in Series 49 as compared with the synthetic optimum diet are not clear especially since this was not true in Series 51.

Summary. The influence of pyridoxine, inositol, and biotin deficiencies on the susceptibility of mice to experimental poliomyelitis has been studied in more than 1400 animals. No striking or consistent difference with reference to susceptibility to either Lansing strain poliomyelitis or Theiler's encephalomyelitis was noted between animals fed diets deficient or optimum in these vitamins.

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The Relative Toxicity of *l*- and *dl*-Serine in Rats.*

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An injurious action of *dl*-serine has been reported previously by the authors.[1,2,3] Thus, the administration of *dl*-serine (100 mg daily) by stomach tube or by injection to rats on certain experimental diets produces anorexia, loss in weight, and albuminuria, leading frequently to death. The most constant pathological finding is the presence of extensive necrotic lesions in the renal tubules. It has been pointed out[2] that the injury may have been due to either one or both of the components of the racemic *dl*-serine. Accordingly, in the present experiments a comparison has been made of the relative toxicity of *l*- and *dl*-serine.‡

Experimental. In most of our previous experiments, rats in groups of 20 were maintained on experimental diets for 4 weeks and received serine (100 mg) daily by stomach tube during the second and the third weeks. In the present study, because of the limited supply of *l*-serine, it would have been impossible to carry on long term experiments on a comparable number of animals so that complete data on weight changes and on mortality§ have not been secured. On the other hand, since the most characteristic clinical and pathological changes develop in the first few days of serine administration, our interest was centered on the alterations appearing during this period, only a few experiments being of longer duration.

In all the present experiments, male rats (100 g) were kept on diet 4 only (no B vitamins),[2] and the amino acid was administered by stomach tube beginning with the eighth day. Of the 8 rats receiving *l*-serine daily in 100 mg amounts, 2 were killed after 1 dose, 3 after 3 doses, 1 after 5 doses, 1 after 9 doses, and 1 a week after the completion of 14 days of serine administration. Usually, for each rat receiving the *l* isomer, another simultaneously received the *dl* compound. The animals were kept in individual metabolism cages. The urine was collected under toluene and was stored in the refrigerator. The urine volume, body weight, and food consumption were recorded daily. The animals were killed by decapitation and the autopsies were performed immediately. The tissues were fixed

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1 Fishman, W. H., and Artom, C., *J. Biol. Chem.*, 1942, **145**, 345.

2 Artom, C., and Fishman, W. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 239.

3 Morehead, R. P., Fishman, W. H., and Artom, C., *Am. J. Path.*, 1945, **21**, 803.

§ No spontaneous deaths occurred in rats receiving *l*-serine.

TABLE I.
Food Consumption and Changes in Body Weight.

	l-Serine Group		*dl*-Serine Group	
	Before Admin.	During Admin.	Before Admin.	During Admin.
Food consumption, g	33; 37; 36; 41; 32	18; 19; 20; 19; 26	37; 28; 42; 28; 29	9; 14; 13; 11; 10
Individual values*	33	27	30	9
Avg	35.3	21.5	32.2	11.0
Changes in body wt, g	+0.5; —1.0; +2.0	—3.5; +1.0; —2.5	—2.0; 0; 0; —2.5	—8.1; —7.2; —7.0
Individual values†	—4.0; —0.5; +6.0	—5.5; +4.0; +9.0	+8.0; +5.0	—10.0; —5.5; —6.5
Avg	+0.5	+0.4	+1.4	—7.4

* Total food consumption of each rat for a 3-day period.
† Increase (+) or decrease (—) in the body weight of each rat for the 3-day period.

in 10% formalin, cut at 6 microns and stained with hematoxylin and eosin.

The urine was tested qualitatively for protein (Heller's, Esbach's, trichloracetic acid, heat coagulation tests) and for reducing sugar (Benedict's test). When the urines reacted positively for protein, its amount was estimated approximately with the quantitative Esbach's test as commonly used clinically.

A more extensive study was made on the urines collected from 2 rats receiving *l*-serine for 3 days and from 2 others receiving *dl*-serine for the same length of time. In these experiments, modifications of published methods were employed for the determination of bisulfite-binding substances,[4] of reducing substances (ceric sulfate titrimetric procedure),[5] of ammonia (steam distillation), and of serine (periodate oxidation).[6] These modifications are described in more detail in the following paper.

Results. The clinical appearance of the animals receiving *l*-serine was uniformly good in contrast to those animals receiving *dl*-serine, which, after 2 or 3 doses of the racemic amino acid, became definitely ill.

Data on food consumption and changes in body weight during the first 3 days of serine administration are recorded in Table I. It appears that the food consumption is reduced in rats receiving both the *l*- and *dl*- compound, but to a smaller extent in the *l*-serine group. No significant change in body weight was observed after the administration of *l*-serine, whereas all the animals receiving the *dl* amino acid showed a marked loss in weight.

The pathological findings in animals receiving *dl*-serine corresponded to those previously described in detail.[3] Severe renal necrosis followed by rapid tubular regeneration and fibrosis, was seen in the kidneys of these animals. In the *l*-serine group, however, there were no demonstrable alterations of the normal renal architecture. Abnormal fatty changes were seen in livers of both groups of animals, but this was to be expected, since the experimental diet was poor in choline or choline precursors.

Protein reactions were all negative or at most doubtfully positive in the urine of rats receiving *l*-serine. On the other hand, in agreement with our previous results,[1,3] significant amounts of protein were present in the urines from rats on *dl*-serine. The proteinuria was most marked during the first 3 days of serine administration, the amounts of protein eliminated in this period ranging between 5 and 13 mg per rat per day.

The findings in the urines of the animals employed in our more detailed investigation are recorded in Table II. In both groups of animals receiving either *dl*- or *l*-serine, the ammonia and the urine volume were increased. After *dl*-serine, in addition to the protein, the urine contained large amounts of unchanged serine for the whole 3-day period. Furthermore, the first day's collection after *dl*-serine showed a marked increase in bisulfite-binding and reducing substances. A strongly positive Benedict's test was obtained on this urine. None of these changes was apparent in the urine of the rats receiving *l*-serine.

Discussion. In the present experiments the

4 Wortis, H., Bueding, E., and Wilson, W. E., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 279.

5 Miller, B. F., and Van Slyke, D. D., *J. Biol. Chem.*, 1936, **114**, 583.

6 Artom, C., *J. Biol. Chem.*, 1945, **157**, 585.

TABLE II.
Analytical Data on Rat Urine.*

	l-Serine Group					*dl*-Serine Group				
	Before admin. 3-day avg	During administration			3-day avg	Before admin. 3-day avg	During administration			3-day avg
		1st day	2nd day	3rd day			1st day	2nd day	3rd day	
Vol., cc	3.7	3.3	5.7	6.7	5.2	3.8	6.3	4.5	8.4	6.4
Proteins (qualitative)	—	±	—	—		—	++	+++	++	
"Sugar" (Benedict's qualitative)	—	—	—	—		—	+++	±	—	
"Serine" (micromoles)	36	42	23	21	29	21	231	250	201	227
NH_3 (micromoles)	166	243	238	199	227	143	197	214	237	216
Reducing substances (glucose equiv. mg)	15	16	13	14	14	15	39	17	11	22
Bisulfite binding substance (micromoles)	27	21	24	19	21	24	55	24	22	34

* Per rat, per day.

same amounts of *dl*- or *l*-serine were given by stomach tube to rats maintained on an experimental diet. Severe clinical disturbances and pathological lesions of the kidney were observed in the animals receiving the racemic mixture. Except for a decrease in appetite (less marked than in the *dl* group), rats receiving the natural isomer did not show injurious effects. It may therefore be reasonably assumed that, under the conditions of our experiments, it is the *d* component which is mainly responsible for these effects.‖

As for the urinary findings, the values in the *l*-serine group (except for NH_3 and urine volume) were almost identical to those found before the administration of the amino acid. On the other hand, in the *dl*-serine group, the urine contained protein, sugar-like substances and larger amounts of substances which bind bisulfite. This last finding may be compared to the observation of an increase of keto-acids (determined as 2,4-dinitrophenylhydrazones) in the urines of rats receiving various racemic amino acids, including *dl*-serine.[7]¶

The greater elimination of unchanged serine after giving the racemic mixture is in line with previous observations on the differences in the utilization of *l*- and *dl*-amino acids observed in humans,[8] in animals,[9] and

‖ One cannot exclude the possibility that the difference noted is one of degree and that some noxious action may become evident, should much larger doses of the *l*-compound be administered. In the present study, the animals received twice as much of the *l* compound as compared with the amount of the *d* component in the racemic mixture.

[7] Waelsch, H., and Miller, H. K., *J. Biol. Chem.*, 1942, **145**, 1.

¶ Waelsch and Miller[7] state: "Only 2 of the 12 amino acids fed as the natural isomers, namely tyrosine and lysine, induced a definite increase in keto acid excretion, whereas all 9 amino acids fed as the racemic compounds did so."

[8] Albanese, A. A., *J. Biol. Chem.*, 1945, **158**, 101; Albanese, A. A., and Frankston, J. E., *J. Biol. Chem.*, 1944, **155**, 101; Albanese, A. A., *Bull. Johns Hopkins Hosp.*, 1944, **75**, 195.

[9] Wohlgemuth, J., *Ber. Chem. Soc.*, 1905, **38**, 2064; Abderhalden, E., and Tetzner, E., *Z. physiol. chem.*, 1935, **232**, 79; Dakin, H. D., *J. Biol. Chem.*, 1910, **8**, 25; Kotake, Y., Matzuoka, Z., and Okagawa, M., *Z. physiol. chem.*, 1922, **122**, 166;

in microörganisms.[10] However, with respect to serine, whereas only the *l* form is utilized by *Lactobacillus*,[11] both *d* and *l* isomers appear to be attacked by *Proteus*,[12] *Pseudomonas*,[13] and *B. coli*.[10,14]

On the basis of the present results, it is not yet possible to decide which one of the general hypotheses, previously suggested,[2] would best explain the injurious action of serine. The present evidence suggests that the *d* isomer is less easily metabolized. Accordingly, one would understand a toxic action due to the increased concentration in the tissues of the unmetabolized *d*-serine (perhaps by a competitive inhibition of certain enzyme systems).

However, according to the alternative hypothesis, the injury may be due instead to products of the metabolism of serine. It may be that *d* serine, although metabolized more slowly, is degraded through a pathway different from that of the natural isomer, giving rise to abnormal toxic compounds.

On the other hand, even if *d*- and *l*-serine are metabolized alike, the normal intermediates may become toxic if they accumulate. The finding of an increased elimination of bisulfite-binding substances in animals receiving *dl*-serine may be pointed out in this connection. One might postulate that after the administration of the unnatural isomer, toxic concentrations of intermediates (such as, possibly, pyruvic acid[14,15,16]) occur in the kidney. This may be the result of an overproduction of the metabolite (*e.g.*, because of the high activity in the kidney of *d*-amino acid oxidase, or other enzymes, specific for *d*-amino acids**) or the consequence of its retarded disposal (*e.g.*, through a diminution in the activity of kidney cocarboxylase).[20]

Summary. A comparison has been made of the relative toxicity of *l*- and *dl*-serine in rats. Under the same experimental conditions, the administration of the natural *l* isomer was not accompanied by the clinical and pathological alterations and by the chemical changes in the urine, characteristically found in animals receiving the racemic mixture. It appears, therefore, that the unnatural *d*-isomer is chiefly responsible for the injurious action of *dl*-serine.

Meeker, E. W., and Wagner, E. C., *Ind. and Eng. Chem., Anal. Ed.*, 1933, **5**, 396; Ratner, S., Schoenheimer, R., and Rittenberg, D., *J. Biol. Chem.*, 1940, **134**, 653; Ratner, S., Weissman, N., and Schoenheimer, R., *J. Biol. Chem.*, 1943, **147**, 549.

10 Gale, E. F., *Bacter. Rev.*, 1940, **4**, 135; Kuiken, K. A., Norman, W. H., Lyman, C. M., and Hale, F., *Science*, 1943, **98**, 266; Kuiken, K. A., Norman, W. H., Lyman, C. M., Hale, F., and Blotter, L., *J. Biol. Chem.*, 1943, **151**, 615; Lewis, J. C., and Olcott, H. S., *J. Biol. Chem.*, 1944, **157**, 265.

11 Stokes, J. L., and Gunness, M., *J. Biol. Chem.*, 1945, **157**, 651.

12 Bernheim, F., Bernheim, M. L. C., and Webster, M. D., *J. Biol. Chem.*, 1935, **110**, 165.

13 Webster, M. D., and Bernheim, F., *J. Biol. Chem.*, 1936, **114**, 265.

14 Chargaff, E., and Sprinson, D. B., *J. Biol. Chem.*, 1943, **151**, 273.

** *d*-serine is acted upon by *d*-amino oxidase at a slower rate than other amino acids,[17] but it is likely that certain *d*-amino acids may be oxidized by different enzyme systems in tissues.[18] The role of the "dehydrase," to which the anaerobic deamination of *dl*-serine by microörganisms and liver has been ascribed,[14,16] remains uncertain. In fact, no specific difference in the action of this enzyme on the *d*- and *l*- component has yet been noted. Moreover kidney slices do not deaminate serine under anaerobic conditions.[19]

15 Bernheim, F., Bernheim, M. L. C., and Gillespie, A. S., *J. Biol. Chem.*, 1936, **114**, 657.

16 Binkley, F., *J. Biol. Chem.*, 1943, **150**, 261.

17 Krebs, H. A., *Biochem. J.*, 1935, **29**, 1620; Klein, J. R., and Handler, P., *J. Biol. Chem.*, 1941, **139**, 103.

18 Karrer, P., and Frank, H., *Helv. Chim. Acta.*, 1940, **23**, 948.

19 Krebs, H. A., *Z. physiol. chem.*, 1933, **217**, 191.

20 Fishman, W. H., and Govier, W., *Science*, 1945, **101**, 77.

15163

Variations of Some Constituents in the Urine of Rats Receiving *dl*-Serine and *dl*-Alanine.*

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In continuation of our investigation on the toxic effects of *dl*-serine and on the mechanism of their production, a more extensive study has now been made of the chemical composition of the urine of rats receiving *dl*-serine by stomach tube. In this regard, we have been interested mainly in the presence of pathological constituents (proteins and sugar-like substances), in the excretion of the administered amino acid and in the variations of certain other compounds or groups of compounds (ammonia, ethanolamine, reducing substances, and bisulfite-binding substances) which may presumably be related to the metabolism or serine.

For purpose of comparison, similar experiments have been performed on rats receiving *dl*-alanine ($CH_3 \cdot CHNH_2 \cdot COOH$), instead of *dl*-serine ($CH_2OH \cdot CHNH_2 \cdot COOH$).

Experimental. In the present experiments, groups of 3 or 4 albino rats, weighing about 100 g, were transferred from the stock diet to diet 4.[1] After 7 days, *dl*-serine (100 mg) was administered once daily to groups *1a* and *1b* and *3* by stomach tube for 12 consecutive days.‡ The animals were then maintained on the experimental diet without serine for 5 more days. In Groups *2a* and *2b*, the administration of *dl*-alanine (100 mg) was substituted for *dl*-serine; otherwise the experimental conditions were identical with those described above. All animals throughout the course of the experiment received a daily injection of a mixture of pure B vitamins,[2] either complete (Group *3*) or without pyridoxine (Groups *1a* and *1b; 2a* and *2b*). Records were kept daily of body weight and food consumption.

The rats (1 or 2 animals per cage) were housed in metabolism cages supported by large glass funnels. In order to collect the urine separately from the feces, these were trapped by a 16-mesh galvanized screen wire in about the middle of the funnel: in addition, glass wool was inserted in the funnel stem. The urine specimens collected under toluene, were removed twice daily and stored in the refrigerator. The 24-hour collections from the rats of each group were pooled. Many of the analyses were performed on these pooled 24-hour collections (especially in the first days of amino acid administration.) Other analyses were done instead on aliquots of 2- to 4-day combined urines.

Methods. The urines were tested qualitatively for sugar-like substances (Benedict's) and for protein. When the urine contained protein, its approximate amount was estimated with the Esbach albuminometer.

"Total amino acids" were evaluated by a formol titration adapted to micro scale, after removal of the proteins, phosphates and carbonates. A description of the method adopted follows:

To 10 cc of urine, diluted to 50 cc, 2 g of permutit are added and the mixture shaken continuously for 5 minutes. The liquid is decanted and the treatment repeated twice. The final mixture is filtered. To a measured amount of the filtrate (*e.g.*, 35 cc) 1 cc of 5% colloidal ferric hydroxide and 3 cc of a 10% $BaCl_2$ solu-

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1 Artom, C., and Fishman, W. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 239.

‡ One death occurred on the 3rd day (Group 3), and another on the 8th day (Group 1a) of serine administration.

2 Fishman, W. H., and Artom, C., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 241.

tion are added. After a short interval, the mixture is alkalinized with an excess of a 3% $Ba(OH)_2$ solution, brought to volume (*e.g.*, 50 cc) and filtered. To an aliquot of the filtrate (*e.g.*, 20 cc) 0.5 cc of a 1% solution of phenolphthalein in 50% alcohol is added, the solution acidified with 0.1 N HCl and the reaction made barely acid to phenolphthalein (approx. pH = 8) with 0.01 N solutions of NaOH and HCl. Fifteen cc of an 18% formol solution (approximately neutral to phenolphthalein) are now added and the mixture titrated with 0.01 N NaOH to a distinct red color (approx. pH = 9.0). A blank containing the same amounts of CO_2-free water, formol, and phenolphthalein is titrated. The volume is equalized by adding the required amount of CO_2-free water and the color of the unknown and blank solutions adjusted to the same tint§ with the standard 0.01 N solutions. The value of the blank is subtracted from the unknown and the results are expressed as micromoles of amino acid excreted per rat per day. Employing the technic as reported here, recoveries of 95 to 100% of added serine and alanine were obtained.‖

"Ethanolamine and unchanged serine" were determined according to a procedure described previously for mixtures of the pure substances in aqueous solution,[3] except that ethanolamine was adsorbed together with ammonia on permutit as described above. The amounts of urine employed were 0.5 to 2.0 cc for the determination of total serine plus ethanolamine and 5 to 10 cc of the permutit filtrate for the determination of serine. When the steam distillation of NH_3, as used in the method, is applied to the urine (or even to the permutit filtrate) in the absence of periodate, appreciable amounts of NH_3 arise from other substances. It is therefore necessary to perform a blank determination in the absence of periodate with every analysis whether on the urine or on the permutit filtrate. The data are expressed as micromoles of serine or ethanolamine excreted per rat per day. Because of the extent of the required corrections, the accuracy of the method as applied to the urine is certainly less than with solutions of the pure substances. However, in testing the technic, 90 to 100% of the ethanolamine and serine added (separately or together) to the urine were recovered.¶

Ammonia was determined in one of two ways. Where analyses were carried out for serine and ethanolamine (Groups *1a, 1b,* and *3*), steam distillation was employed as indicated above. The ammonia artificially formed during the 6-minute period of distillation was evaluated by continuing the process for a second 6-minute period, and the value so obtained was used in correcting the ammonia values of the first 6-minute period. In Groups *2a* and *2b,* a formol titration was carried out according to the procedure described above, except that the treatment with permutit was omitted and the titration values were increased by a 3% correction. The difference between the values obtained with the formol titration before and after absorption was considered to represent the ammonia. When both ammonia procedures were applied to the same urine, satisfactory agreement was obtained. The values are expressed as micromoles of ammonia per rat per day.

Reducing substances. 1.0 cc of urine was diluted with H_2O to 10 cc and 1 cc of 30% NaCl solution and 1.0 cc of 5% colloidal ferric hydroxide were added. The mixture was brought to 15 cc with distilled H_2O and filtered after 5 minutes. 5.0 cc of the filtrate were made alkaline and the reducing power was determined according to the method of

§ In most cases, the final filtrates of the diluted urine after neutralization are practically colorless. Occasionally the use of a Walpole comparator was required to compensate for the slight residual color.

‖ Formol titration values always have a somewhat arbitrary significance. Our values probably include, in addition to the amino acids, lower polypeptides, and possibly amino bases, weakly dissociated, which might have escaped adsorption by permutit. Likewise, the possibility that traces of ammonia are also included cannot be ruled out completely.

3 Artom, C., *J. Biol. Chem.*, 1945, **157**, 585.

¶ Obviously, the serine figures may include other hydroxyamino acids, and the ethanolamine data, other non-volatile (relatively strong) bases with adjacent amino and hydroxy groups, which might have been adsorbed on permutit.

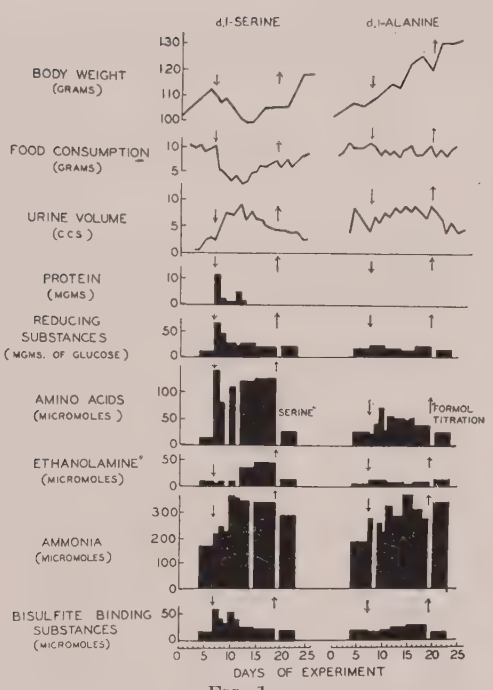

FIG. 1.

The variations in body weight, food consumption, volume and composition of the urine of rats to which either *dl*-serine or *dl*-alanine (100 mg per rat per day) was given by stomach tube. The arrows indicate the day on which the administration of the amino acid was started (↓), and that on which it was discontinued (↑). All rats were on an experimental diet (Diet 4) and received a daily injection of a mixture of pure B vitamins without pyridoxine.

Miller and Van Slyke.[4] The data are expressed as milligrams of glucose per rat per day.

Bisulfite-binding substances were determined on 2.5 cc of the urine which had been acidified with 1 cc of a 3% HCl solution and diluted with H_2O to 20 cc. The analyses were completed as described by Wortis, Bueding, and Wilson.[5] Two equivalents of I_2 are required for each free =CO or —CHO group. The results have been expressed accordingly as micromoles of carbonyl compound excreted per rat per day.

Results. In Fig. 1, data obtained from Groups *1a* and *1b,* receiving *dl*-serine, have been averaged and compared with similar data from Groups *2a* and *2b,* receiving *dl*-alanine.

In agreement with our previous results,[1,2] the administration of *dl*-serine causes a characteristic fall in food consumption and in body weight during the first week. This is followed by a partial recovery of growth and appetite in spite of the continued administration of the amino acid. In the case of the *dl*-alanine groups, there is no significant change in either the food consumption or the rate of growth upon administration of the amino acid.

As for the urinary findings in the animals of the serine group, some of the present results confirm and extend those of the preceding paper.[6] It should be remembered that the rats used in that investigation did not receive B vitamins.

The volume of urine eliminated after giving serine is probably increased with a maximum value at the fourth day, whereas little or no change was observed in the urine volume of the rats receiving *dl*-alanine.

Qualitative reactions for protein and sugar-like substances were positive in the first few days of serine administration and were negative in the alanine group. Evidence that glucose was present in the urine of the serine groups is supplied by the following findings: (1) Formation of CO_2 by baker's yeast. In the urine after fermentation, the Benedict's test became negative. (2) Formation of typical crystals of glucosazone in rather large amounts by reaction with phenylhydrazine. The osazone, recrystallized from alcohol, melted at 198°-199° (uncorrected). No depression of the melting point was observed upon admixture with an authentic sample of glucosazone.

As for the excretion of the amino acids, a substantial proportion (6-13%) of the serine administered is eliminated unchanged throughout the period of administration. On the other hand, on the basis of the formol titration values, much less of the *dl*-alanine administered (4% at most) is apparently eliminated as such. A significant increase in substances behaving as ethanolamine was detected only

[4] Miller, B. F., and Van Slyke, D. D., *J. Biol. Chem.*, 1936, **114**, 583.

[5] Wortis, H., Bueding, E., and Wilson, W. E., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 279.

[6] Artom, C., Fishman, W. H., and Morehead, R. P., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 284.

during the second week of serine administration.

There is probably an increase of ammonia after giving each amino acid. The amounts of reducing and bisulfite-binding substances after serine were both markedly elevated in the first days of serine administration, whereas no change was observed after giving *dl*-alanine.

The experimental data of Group *3* (receiving B vitamins plus pyridoxine and *dl*-serine) have been omitted for the sake of brevity. The findings were substantially the same as described for Groups *1a* and *1b*, except that the elimination of bisulfite-binding substances, of ammonia and of serine was more irregular and reached maximum values somewhat later than in Groups *1a* and *1b*.

In some urines, simultaneous determinations of total amino acids and of serine were performed. The differences in the two (which presumably represent amino acids other than serine) were not more than 40 micromoles per rat per day before administering the amino acids. Values found in the urine of rats receiving alanine or serine were often of the same order of magnitude. However, occasionally, and especially in Group *3*, much higher values were found after giving *dl*-serine (83 micromoles on the 1st, 100 micromoles on the pooled urine collected on the 6th, 7th, and 8th days of serine administration).

Discussion. The results of the present investigation corroborate the statement[6] that there are certain characteristic findings in the urine of the animals receiving *dl*-serine. These findings have not been obtained in other animals receiving *dl*-alanine under the same conditions: a point which is interesting in view of the similarity in structure of these 2 amino acids. In this connection, it may also be mentioned that the kidneys of animals receiving *dl*-alanine (studied by Dr. R. P. Morehead) showed no demonstrable pathological lesions.**

Some of the findings, such as the proteinuria, are probably the consequence of the kidney lesions which are already apparent after the first dose of *dl*-serine.[10] The simultaneous presence of sugar might be interpreted similarly. In this respect, one would be tempted to think of a rather specific action of serine on the anatomical structures or on the physiological mechanisms involved in the reabsorption of glucose from the glomerular ultrafiltrate.

In view of the results of the preceding paper, the values for urinary serine obtained in the present study represent probably the elimination of *d*-serine. Consequently, it appears that quite a large fraction (12-25%) of the unnatural isomer has not been metabolized. It has already been pointed out[6] that this fact would be in line with the interpretation of serine injury as being due to a mass action effect of the unmetabolized *d*-serine. However, if this be true, one would expect the clinical recovery to be accompanied by a greater utilization of the administered serine. This expectation is not borne out by the results of the present experiments, in which the excretion of serine persisted at a high level throughout the entire period of the experiment.

Evidence for the formation of some ethanolamine in the body by the decarboxylation of serine has been reported.[11] In the present study, significant amounts of substances reacting as ethanolamine have been found in the urine of the serine-treated animals. This finding occurs rather late (that is, in the period of the experiment when the animals show signs of clinical recovery), and therefore it is not in favor of the possibility that ethanolamine is the agent responsible for the injurious action of serine. In any case, the action of

** *dl*-alanine has been shown to be toxic in pigeons.[7] The action appears to be due to the *d* component.[8] Noxious effects from *d*-alanine have also been observed in rats and guinea pigs. However, in these animals much larger doses are required to produce these effects (700 mg of *d*-alanine per 100 g body weight).[9] Such amounts are 14 times as great as the quantities employed in the present experiments.

[7] Lombroso, U., *Boll. Soc. Ital. Biol. Sp.*, 1933, **8**, 362.

[8] Abderhalden, E., and Tetzner, E., *Z. physiol. Chem.*, 1935, **232**, 79.

[9] Edlbacher, S., and Wiss, O., *Helv. Chim. Acta.*, 1944, **27**, 1060.

[10] Morehead, R. P., Fishman, W. H., and Artom, C., *Am. J. Path.*, 1945, **21**, 803.

[11] Stetten, D., *J. Biol. Chem.*, 1942, **144**, 501.

the intestinal flora does not appear to be essential.[1]

Concerning the increase in bisulfite-binding substances, as suggested previously,[6] it is likely that these substances may include certain products of the metabolism of serine, such as pyruvic acid.[12,13,14] This compound is also formed in the oxidative deamination of alanine. However, no increase in bisulfite-binding substances was found after giving *dl*-alanine.†† This makes less probable the hypothesis of an overproduction of pyruvic acid (*e.g.*, by kidney *d*-amino acid oxidase) as an explanation for the increase in bisulfite-binding substances. Therefore, the hypothesis of a defect in the disposal of pyruvic acid (*e.g.*, as a result of a diminution in kidney cocarboxylase[16]) seems more attractive.

It is hardly necessary to point out that many interpretations other than the ones mentioned are possible; for example, an abnormal concentration of pyruvic acid may arise from an impaired metabolism of carbohydrates and not from serine.

The occasional finding of significant amounts of amino acids other than serine in the urine of animals receiving this amino acid may be of interest. In this connection, one may mention the hypothesis of the formation of glycine in the course of the metabolism of serine,[17,18,19] for which more direct evidence has been presented recently.[20] Another possibility is the production of cystine (or cysteine) from serine.[21,22]

It is difficult to state to what extent the present results explain the mechanism of serine injury. However, as mentioned above, certain findings (protein, sugar, increase in bisulfite-binding and reducing substances) which occur early in the period of serine administration may reasonably be related to the lesion (either as its cause or as its effect), and those findings occurring later (such as the production of ethanolamine) can be thought of as being associated with the recovery of the animal.

Summary. The variations of some chemical constituents in the urine of rats receiving *dl*-serine have been studied quantitatively. The results have been compared with those of similar experiments in which *dl*-alanine has been given. In the serine groups, a significant proportion of the administered amino acid is excreted in the urine. In addition, in the first few days of serine administration, the urine contained protein and sugar, and the amounts of bisulfite-binding and of reducing substances were elevated. The presence of ethanolamine in later periods of the experiment appears probable. None of these changes was observed in the urine of animals receiving *dl*-alanine.

The possible bearing of these results to the injurious action of serine is discussed. At any rate, the present data confirm and extend the indication of chemical changes in the urine which are as typical as the other clinical and pathological alterations found in rats receiving *dl*-serine.

12 Bernheim, F., Bernheim, M. L. C., and Gillespie, A. G., *J. Biol. Chem.*, 1936, **114**, 657.

13 Chargaff, E., and Sprinson, D. B., *J. Biol. Chem.*, 1943, **151**, 273.

14 Binkley, F., *J. Biol. Chem.*, 1943, **150**, 261.

†† Likewise, Waelsch and Miller[15] did not find any increase in urinary keto acids after giving *dl*-alanine.

15 Waelsch, H., and Miller, H. K., *J. Biol. Chem.*, 1942, **145**, 1.

16 Fishman, W. H., and Govier, Wm., *Science*, 1945, **101**, 277.

17 Dakin, H. D., *J. Biol. Chem.*, 1909, **6**, 235.

18 Knoop, F. Z., *Z. physiol. Chem.*, 1914, **89**, 151.

19 Leuthardt, F., and Glasson, B., *Helv. Chim. Acta.*, 1942, **25**, 245.

20 Schemin, D., *Federation Proceedings*, 1945, **4**, 103.

21 Toennies, G., *J. Biol. Chem.*, 1940, **132**, 455.

22 Binkley, F., and Du Vigneaud, V., *J. Biol. Chem.*, 1942, **144**, 507.

15164 P

Colorimetric Estimation of Penicillin in Aqueous Solutions.*†

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It was recently shown that acid hydrolyzates of penicillin gave a blue-violet coloration with the ninhydrin reagent.[1] Moreover, the same workers reported that the color intensity of this reaction ran parallel to the antibacterial activities of their preparations.

Colorimetric analysis of amino acids by the ninhydrin reagents has been reported by other investigators.[2,3]

The writer has modified the ninhydrin reagents so that small amounts of crystalline penicillin can be estimated with fairly consistent results, provided that temperature, duration of heating, and pH are rigorously controlled. These experiments have been performed on aqueous solutions of crystalline penicillin (Merck), with potency of 1650 units/mg.

The procedure is as follows:

To graduated 10 cc tubes are added 3 to 9 μg of freshly diluted solution of penicillin, 0.5 cc of 0.2 N H_2SO_4, 4 mg of ninhydrin (Eastman) treated with norite (Coleman and Bell). The tubes are then covered with glass vials and heated in boiling water bath for 20 minutes in subdued light. The tubes are then removed, cooled and treated with 0.2 cc of freshly prepared 10% pyridine (Mallinckrodt), 20 mg of norited $(NH_4)_2SO_4$ (Baker)† the volumes equalized with distilled water and then reheated for 15 minutes. The tubes are removed, cooled and made up to 6 cc with water.

The pH of these solutions should be from 4.7 to 5.0. They are then read in the Coleman spectrophotometer against reagent blanks at 550 μ. Blanks and penicillin solutions are prepared in triplicate.

The actual values obtained are not absolute but vary somewhat with the reagents and experimental conditions. However, straight line calibrations (semi-log)) may be obtained for any one set of reagents and conditions.

An illustrative set of values is as follows:

Concentration penicillin, gamma	Light transmission, %
3	88.5
6	79
9	71.5

Summary. A colorimetric technic for analysis of 9 μg or less of crystalline penicillin is described.

* The writer is indebted to Merck & Co., Rahway, N.J., for their generous gift of penicillin.

† Research paper No. 579, Journal Series, University of Arkansas.

1 Abraham, E. P., Chain, E., Baker, W., and Robinson, R., *Nature*, 1943, **151**, 107.

2 Harding, V. J., and Maclean, R. M., *J. Biol. Chem.*, 1915, **20**, 217.

3 Virtanen, A. I., and Laine, T., *Skand. Arch. f. Physiol.*, 1938, **80**, 392.

† 25 cc of 20% $(NH_4)_2SO_4$ is shaken with 250 mg norite and filtered.

15165

Parasympathomimetic Effect of Aqueous Humor in Human Eyes with and Without Chronic Simple Glaucoma.

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Velhagen[1] demonstrated that the aqueous humor of certain animals produced a sympathomimetic effect on the isolated frog heart. Engelhart[2] one year later instilled eserine in the eyes of rabbits to prevent the destruction

* Theodore Escherich Research Fellow.

1 Velhagen, K., *Arch. f. Augenheil.*, 1930, **103**, 424.

2 Engelhart, E., *Arch. f. d. ges. Physiol.*, 1931, **227**, 220.

by cholinesterase of any acetylcholine present, and then exposed the eyes to light for parasympathetic stimulation. Under these conditions he proved the presence of acetylcholine in the aqueous humor. Pletneva, Raeva, and Voronina[3] studied the biological effect of the aqueous humor of human eyes and found that a sympathomimetic substance was present in some and a parasympathomimetic substance in others. They could demonstrate no consistent effect on the isolated frog heart by the aqueous in normal eyes or in any of the pathological ocular conditions they investigated. Apparently they did not use eserine or the standardized exposure to light according to the Engelhart technic.

The purpose of this study was to determine quantitatively the acetylcholine content of the aqueous humor of human eyes prepared according to the method applied by Engelhart to rabbits. Furthermore, since there is clinical evidence to suggest a deficiency of acetylcholine in glaucoma, determinations were made on a series of eyes with chronic simple glaucoma, and on a control group without history or signs of increased ocular tension.

Each eye to be studied was prepared by the instillation of 1 drop of 1% eserine salicylate into the conjunctival sac 3 times at 10-minute intervals beginning 1½ hours before operation. No other eye drops were used for 24 hours preceding the experiment, nor was systemic medication permitted. For 20 minutes to one-half hour before the procedure the bright Hammer lamp of the operating room was focused on the subject eye, with winking permitted. In this way, reflex stimulation of the parasympathetic nerves to the eye was produced. Anesthesia was obtained by retrobulbar injection of 2 cc of 4% novocaine. Preliminary studies on rabbits had shown that such procedure did not produce a demonstrable change in the biological effect of the aqueous humor. The conjunctival instillation of local anesthetic solutions was avoided since their passage into the aqueous might influence the results of the bioassay. Approximately 0.1 cc of primary aqueous humor was obtained from each eye, by paracentesis and aspiration with a hypodermic needle attached to a tuberculin syringe.

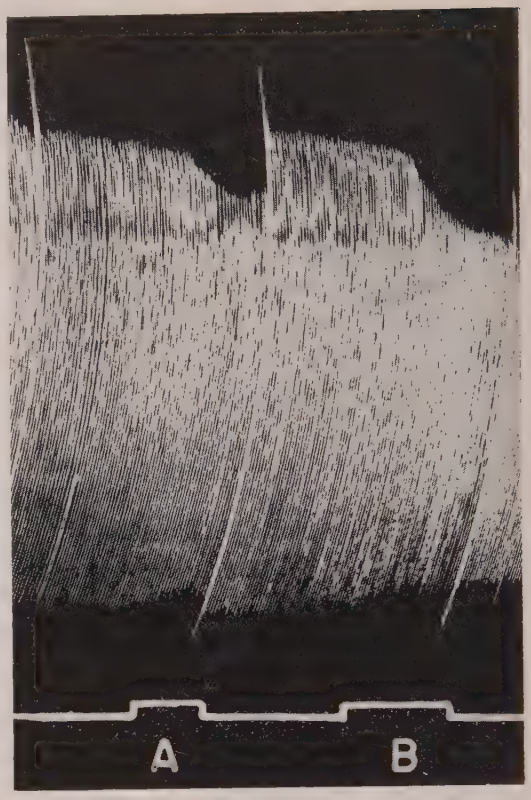

FIG. 1.
Kymographic record of response of frog heart preparation to aqueous humor from non-glaucomatous eye with diabetic cataract. A: Effect of 0.1 cc of undiluted aqueous humor. B: Effect of 0.1 cc of acetylcholine 1:100,000,000 on same preparation.

The effect of each sample was tested within 30 minutes on the freshly prepared isolated heart of the spring or summer frog (*Rana pipiens*) according to the perfusion method of Straub.[4] These preparations were usually found to be sensitive to acetylcholine in dilutions up to 1:1 billion. The aqueous was diluted 1:6 by the perfusion fluid to which it was added. A continuous kymographic record of the heart beat was made during each experiment. For an approximately quantitative estimation, comparisons were made between the effects of the aqueous humor samples

[3] Pletneva, N., Raeva, N., and Voronina, E., *Vestnik Oftal.*, 1938, **13**, 462.

[4] Sollman, T., *Laboratory Guide in Pharmacology*, p. 190, W. B. Saunders Co., Phila., 1917.

FIG. 2.

Inhibition of parasympathomimetic effect of aqueous humor by atropine sulphate. A: Effect of 0.08 cc of aqueous humor from normal eye. B: Addition of 0.1 cc of 1:10,000 atropine sulphate to perfusion fluid. C: Subsequent effect of 0.08 cc of same sample of aqueous humor as A.

and those of known dilutions of acetylcholine chloride on the same hearts.

Ten eyes with various non-inflammatory pathological conditions, but without glaucoma, and 7 eyes with chronic non-congestive glaucoma, not controlled by miotics, were studied. Two eyes in the latter group had previously undergone iridectomy without apparent effect on the disease. The aqueous humor of the 10 non-glaucomatous eyes produced an inotropic depression of the heart beat in each instance. In 8 of these experiments the intensity of this parasympathomimetic effect was approximately equal to that produced on the same heart by a similar amount of acetylcholine in 1 to 100 million dilution. The record of one experiment of this type is reproduced in Fig. 1. In the remaining 2 assays in this group the depression produced was sufficient to cause diastolic standstill for several seconds followed by resumption of the normal beat. This effect could be reproduced with known concentrations of acetylcholine of over 1 to 1,000,000.

Enough aqueous humor was obtained from 2 of these non-glaucomatous eyes to permit the testing of 2 samples from each. After one-half of the aqueous had been demonstrated to cause a depression in amplitude of the heart beat, 0.1 cc of 1 to 10,000 atropine sulfate solution was added to the cannula and the second half of the aqueous tested. In both cases complete inhibition of the parasympathomimetic effect previously noted occurred. The record of one of these experiments is shown in Fig. 2. Since it is characteristic of the pharmacological effect of acetylcholine that it is inhibited by atropine, this demonstration strongly suggests that the parasympathomimetic agent in the aqueous is similar to or identical with acetylcholine.[5]

The aqueous humor of each of the 7 eyes with chronic simple glaucoma was similarly tested. In no instance did a sample from these eyes produce a detectable parasympathomimetic depression of the cardiac contractions. The effect of the aqueous of an eye with chronic simple glaucoma is reproduced in

FIG. 3.

Kymographic record of response of frog heart preparation to aqueous humor from eye with chronic simple glaucoma. A: Effect of 0.25 cc of acetylcholine 1:100,000,000 to demonstrate sensitivity of preparation. B: Effect of 0.1 cc of aqueous humor from glaucomatous eye. C: Effect of 0.1 cc of acetylcholine 1:100,000,000.

[5] Chang, H. C., and Gaddum, J. H., *J. Physiol.*, 1933, **79**, 255.

Fig. 3. In the bioassay of the aqueous humor from 2 of these eyes, a slight augmentative effect was noted. It thus appears that in contrast to the aqueous of non-glaucomatous eyes, no acetylcholine-like substance is demonstrable in the aqueous humor of eyes with chronic simple glaucoma when tested by this method.

Conclusions. The results are presented of the bioassay of aqueous humor from human eyes prepared according to the method applied by Engelhart to animals. In a series of 10 human eyes without glaucoma, every sample of aqueous humor contained a parasympathomimetic substance which, in the 2 cases so tested, was found to be similar to acetylcholine. In the aqueous humor of 7 human eyes with chronic simple glaucoma, no such parasympathomimetic substance could be demonstrated.

The author wishes to express his appreciation to Dr. Robert K. Lambert for his cooperation, and to Miss Mary E. Carsten for technical assistance in this work.

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Renal Clearance of β-Hydroxybutyric Acid in a Dog.

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Urinary excretion of β-hydroxybutyrate (βB) commonly occurs in ketosis of starvation and diabetes and may be induced experimentally by injections of anterior pituitary extracts or of certain of the ketone bodies and fatty acids. Evidence has been obtained from studies on rats[1] and humans[2] which indicates that excretion of total ketone bodies increases markedly when their concentration in the blood rises above 15 to 20 mg % in humans and above 25 to 30 mg % in rats. However, variable amounts of acetone and acetoacetate are found associated with βB and analyses of total ketone bodies cannot yield conclusive information regarding excretion of βB. Intravenous injection of 0.5 g of βB per kilo per hour into a dog was found necessary to cause an appreciable excretion of βB. At a rate of 0.4 g per kilo per hour only slight amounts of βB were found to have been excreted. None was found in the urine when the rate of infusion was 0.3 g per kilo per hour.[3]

Procedure. The renal clearance of *dl*-βB was investigated in 2 experiments on a trained female dog weighing 13.6 kg. In the first experiment an intravenous infusion of 1.9 cc per minute of a 7.5% solution of sodium *dl*-βB and 1% creatinine was maintained for 45 minutes, following an initial priming injection of 3 g of sodium *dl*-βB and 1.5 g creatinine. Creatinine was infused to permit the measurement of the glomerular filtration rate. About 500 ml of water were given by stomach tube 45 minutes before the infusion was begun. In a second experiment 13 g of sodium *dl*-βB in 25% solution were given in a single initial intravenous dose. Creatinine, 100 mg per kg, was given subcutaneously 30 minutes before collection of urine samples. Diuresis was maintained with water given at 45-minute intervals. Because of the difficulty of obtaining the compound, the time allowed in each experiment for mixing was shortened to 12 minutes. The levels of creatinine and βB were followed in each experiment for about 1.5 hours with urine collections approximately every 10 minutes and blood samples every 15 minutes.

Analyses. The plasma proteins were precipitated by $CdSO_4$.[4] The carbohydrates in

[1] Shipley, R. A., and Long, C. N. H., *Biochem. J.*, 1938, **32**, 2242.

[2] Martin, H. E., and Wick, A. N., *J. Clin. Invest.*, 1943, **22**, 235.

[3] Wilder, R. M., *J. Biol. Chem.*, 1917, **31**, 59.

[4] Fujita, A., and Iwatake, D., *Biochem. Z.*, 1931, **242**, 43.

60 ml of filtrate were precipitated by the addition of 6 ml of 25% $CuSO_4$ and an excess of dry $Ca(OH)_2$.[5] The carbohydrates in 30 ml of urine were precipitated by addition of 15 ml of 25% $CuSO_4$ and 15 ml of 10% lime suspension. The Cu-lime filtrate acidified with 0.5 cc of 50% H_2SO_4 was freed of acetone and acetoacetic acid by boiling for 5 minutes without reflux. The method of Barnes and Wick was modified by addition of approximately 0.25 g of ferrous alum to the Hg precipitate before distillation to prevent formation of free chlorine. Creatinine was determined[6] in the Cd plasma filtrates and in diluted urines.

Results. In the first experiment the plasma level of βB was maintained at nearly 40 mg % with only a slight fall during the infusion, after which the level fell to 6 mg % one hour after the infusion was shut off. The rate of tubular reabsorption of βB ($T_{\beta B}$) during the first 3 ten-minute periods was between 30 and 35 mg per minute when the plasma level was between 40 and 45 mg % and gradually fell to 5 mg per minute when the plasma level was 8 mg %. The load/$T_{\beta B}$ (plasma concentration × filtration rate/rate of tubular reabsorption) ratios were between 1.1 and 1.2 during the first 3 periods. Reabsorption of βB approached but did not reach completeness as the plasma level fell to 8 mg %. At this level reabsorption was incomplete by only 2%, while at a plasma level of 21 mg % reabsorption failed of completeness by 9%.

In the second experiment the plasma concentration of βB was 95 mg % 15 minutes after injection and the level fell to 32 mg % during the following 69 minutes. The rate of tubular reabsorption ($T_{\beta B}$) ranged between 28 and 38 during the 5 periods when the plasma level of βB was above 40 mg %. The load/$T_{\beta B}$ ratios ranged from 1.3 to 2.0 during these periods. At a plasma level of 35 mg % reabsorption was only 80% complete while at a plasma level of 96 mg % reabsorption was 50% complete. The maximum value ($Tm_{\beta B}$) for tubular reabsorption would appear to lie between 35 and 40 mg per minute for a dog weighing 12 to 15 kg.

Discussion. The finding of a gradual rather than a precipitate increase in the rate of excretion by the kidney of *dl*-βB with increasing plasma levels would not be anticipated from previous work on ketonuria. However, it is in accord with recent work on the excretion of amino acids[7] and of lactic acid (F. N. Craig, unpublished). The failure of reabsorption of 2% of the βB in the tubular filtrate, as occurs at a plasma level of 8 mg %, involves the loss of about 10 mg of βB per hour by a 15 to 20 kilogram dog. This is a not inconsiderable amount since at ordinary urine pH values it involves the loss of approximately 10 m.eq. of sodium. At higher plasma levels the rate of loss would lead increasingly rapidly to acidosis, unless sodium was supplied in a suitable form. For example, at a plasma level of 35 mg % 20% fails of reabsorption, leading to excretion of βB at the rate of 400 mg per hour, equivalent to approximately 400 m.eq. per hour, by a 15 to 20 kg dog.

Conclusion. Two renal clearance experiments in a dog indicate that *dl*-β-hydroxybutyrate is excreted by the kidney in a fashion resembling many amino acids. The maximum rate of tubular reabsorption ($Tm_{\beta B}$) appears to lie between 2 and 3 mg per minute per kg of animal. An appreciable excretion of β-hydroxybutyrate occurs at a plasma level of 8 mg %.

Acknowledgment is made to Dr. Homer W. Smith for criticism of the manuscript.

[5] Barnes, R. H., and Wick, A. N., *J. Biol. Chem.*, 1939, **131**, 413.

[6] Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, **38**, 81.

[7] Pitts, R. F., *Am. J. Physiol.*, 1943, **140**, 157.

15167

Brain Abscess and Meningitis Caused by a Diphtheroid Resembling *Corynebacterium ulcerogenes.*

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Rare cases of meningitis and brain abscesses caused by other organisms than the meningococcus, pneumococcus, streptococcus, tubercle bacillus and Pfeiffer's bacillus have been reported from time to time. Topley and Wilson[1] mention *B. anthracis, E. coli,* and Friedlander's bacillus among others as an infrequent cause of meningitis. *Actinomyces bovis* has been found in an abscess of a child's brain,[2] and *Brucella suis* was incriminated by Hansmann and Schenchen.[3] *Bacterium enteritidis,*[4] as well as *Neisseria gonorrheæ,*[5] has been shown to be the cause of meningitis. In 1936, a filterable virus was suggested as the cause of suppurative meningitis.[6] Recently the supposedly non-pathogenic *S. marcescens* has been isolated from a case of meningitis.[7]

There are relatively few references in the literature to diphtheroids as the cause of meningitis. Two such cases have been reported[8,9] in which the causative organism was similar to the one herein described with respect to pathogenicity to laboratory animals, but was different in certain other important respects.

Kessel and Romanoff reported an organism which they classified as *Corynebacterium hoffmanni* as the cause of a case of meningitis.[10] Schultz, Terry, Bruce and Gebhardt[11] reported the isolation of an organism from a case of meningoencephalitis which they named *Corynebacterium parvulum,* but which later proved to be *Listerella monocytogenes.* This bacterium was fatal within 72 hours when given intravenously or intracerebrally to rabbits, mice, guinea pigs, and rhesus monkeys. Gibson[12] isolated a diphtheroid bacillus from a fatal case of meningitis which was highly virulent for guinea pigs, rabbits, rats, and mice when given intraperitoneally or intravenously.

Experimental. The organism to be described in this paper was isolated repeatedly in pure culture from a brain abscess and spinal fluid of a 7-year-old child who was under observation from December, 1943, to July 1, 1944, at which time she expired.

Pathology. Microscopic sections of the brain tissue showed only necrosis and a non-specific inflammatory reaction.

Bacteriology. Smears made from the abscess pus repeatedly showed the presence of organisms having the characteristics described below. No organisms were seen in eosin-hematoxylin stains of histological sections.

Aerobic and anaerobic cultures as well as guinea pig inoculations were made, using the pus from the brain abscess and spinal fluid. The guinea pigs remained normal. The only organism isolated was found growing aero-

1 Topley and Wilson, *Principles of Bacteriology and Immunology,* 2nd Ed., Wm. Wood and Company, p. 1146.

2 D'Ewart, John and Dawson, George D., *Brit. Med. J.,* 1927, **1**, 718.

3 Hansmann, G. H., and Schencken, J. R., *Am. J. Path.,* 1932, **8**, 435.

4 Stevenson, F. N., and Wells, L. K., *Lancet,* 1933, **225**, 1084.

5 Strumia, M. M., and Kohlhas, J. J., *J. Inf. Dis.,* 1933, **53**, 212.

6 Rivers, T. M., and Scott, T. F. M., *Science,* 1935, **81**, 439.

7 Aronson, J. D., and Alderman, *J. Bact.,* 1943, **46**, 261.

8 Dick, G. F., *J. A. M. A.,* 1920, **74**, 84.

9 Miller, M. K., and Lyon, M. W., Jr., *Am. J. M. Sc.,* 1941, **162**, 593.

10 Kessel, Leo, and Romanoff, Alfred, *J. A. M. A.,* 1930, **94**, 1647.

11 Schultz, E. W., Terry, M. C., Bruce, A. T., Jr., and Gebhardt, L. P., PROC. SOC. EXP. BIOL. AND MED., 1933, **31**, 1021.

12 Gibson, H. J., *J. Path. and Bact.,* 1935, **41**, 239.

bically, and was a Gram positive rod resembling those of the genus Corynebacterium.

The organism isolated repeatedly from the patient showed the following:

Morphological and Staining Characteristics. When grown on blood agar for 48 hours at 37°C and stained with methylene blue, rods varying from 1 to 5 μ long by 0.5 to 1 μ wide were observed. Some curved and coccoid forms were present, but most were straight rods. Clubbing was observed, the clubs appearing at one end only. Two to 3 beads were seen in many of the bacteria. The appearance closely resembled the true diphtheria bacillus. However, no palisade formation and but few V and W forms were observed.

Smears made from Loeffler's medium and stained with methylene blue were more uniform in size than were those from the blood agar. The coccobacillary type predominated and there was no clubbing. Few granules were in evidence. Gram stains of smears from cultures on Loeffler's medium were Gram positive. A tendency to be in parallel pairs was noted in this and other stains. This seems quite characteristic for the organism. Neisser's stain showed nothing more than did methylene blue.

A Gram stain of the surface growth of a 48-hour Veillon agar stab showed coccobacillary forms predominating, many of which were in pairs. Granules at both ends of the rods were observed in many. The majority of the organisms retained the stain. A stain made from organisms taken from the bottom of the stab showed more rod-like bacteria, larger than those taken from the top, and with more clubbing and more bizarre forms in evidence.

The stained organism appeared about the same when grown on plain agar as when grown on Loeffler's medium. They are not acid fast, non-motile, non-capsule forming, and do not form spores.

Growth Characteristics. This organism is a facultative anaerobe with an optimum temperature for growth at 35-37°C, but will grow scantily at room temperature. It is not fastidious in its nutritive requirements and grows on all of the ordinary laboratory media. Growth was rather scant on plain agar after 24 hours, but in 48 hours, it was good. The colonies are small, round, and white. Microscopically they appear to have a granular surface. The edges are smooth and entire. There was no apparent change in colony formation after being transferred every week for several months on nutrient agar slants.

Colonial growth on Loeffler's medium was about the same as on plain agar, with pinpoint, discrete, white, entire colonies being produced. On blood agar, however, a somewhat larger colony was produced. These colonies are greyish white, moist, convex, and of a butyrous consistency. No zone of hemolysis was seen. Under low power the colonies appeared round and smooth. The colonies on chocolate agar showed little difference from those on whole blood agar. Tellurite agar allowed only slight growth, but the colonies were definitely blackened.

A Veillon agar stab was made which showed a thin, filmy villous growth along the stab in 48 hours. The growth became less toward the bottom of the tube. On gelatin a scant growth was seen on the surface after 9 days and no liquefaction took place.

Growth in plain broth was scant in 24 hours, but good in 48 hours. The broth was turbid with a stringy sediment. On first isolation in peptone media, no growth was seen. However, growth was obtained in this medium in 48 hours after the bacterium had been growing on other media in the laboratory for several months. Tryptose phosphate broth produced a cloudy, stringy growth in 24 hours at 37°C. A synthetic broth composed of glucose, $NaNH_4HPO_4$, $MgSO_4$, and K_2HPO_4 would not support visible growth. Stained smears showed swollen, oval, and other pleomorphic forms.

Biochemical Reactions. No indole was produced from peptone and nitrates were not reduced. The methyl red and V.P. tests were negative. Catalase was produced after 48 hours. Lead acetate showed no blackening. No active dehydrogenases for lactic acid, glucose, maltose, sucrose, or glycerol could be demonstrated. However, methylene blue was reduced in nutrient agar in 18 hours.

Litmus milk was neither coagulated, re-

TABLE I.

	Sulfanilamide				Sulfamerazine				Sulfasuxadine				Sulfaguanidine			
Mg/ml	.25	.12	.06	.006	.25	.12	.06	.006	.25	.12	.06	.006	.25	.12	.06	.006
24 hr	—	—	+	+	—	—	—	+	—	—	+	+	—	+	+	+
48	—	+	+	+	—	—	—	+	—	+	+	+	—	+	+	+
72	—	+	+	+	—	—	+	+	—	+	+	+	—	+	+	+
96	—	+	+	+	—	—	+	+	—	+	+	+	+	+	+	+

\+ equals growth as determined by visible cloudiness
— equals no growth.

duced, nor was acid or base formed in 10 days. Of the carbohydrates, dextrose, and levulose alone were fermented, producing acid but no gas. Sucrose, lactose, maltose, galactose, salicin, dextrin, mannitol, and glycerin were not attacked. Starch agar plates when treated with iodine showed a reddish to clear zone around most of the colonies.

Inhibiting Agents. Penicillin produced in our laboratory when added to nutrient broth in a concentration of 6 units per ml, failed to inhibit the growth of this organism, while 0.03 unit per ml inhibited the growth of staphylococci.

Neither did 7 mg per ml of sulfanilamide nor 1 mg per ml of sulfaguanidine inhibit growth when added to nutrient broth. However, using peptone as the test medium, the results in Table I were observed.

All 4 of the sulfa drugs dissolved in peptone broth in a concentration of 0.25 mg per ml inhibited the growth of the organism for at least 72 hours. Sulfamerazine was the most effective and sulfaguanidine the least effective.

Pathogenicity for Animals. Intracerebral inoculation of mice with 0.03 ml of a 24-hour broth culture of the growing organisms produced no symptoms. The brains were removed after 3 weeks and no organisms could be demonstrated. The brains appeared to be normal. Similar results were obtained when .15 ml of the organism suspended in saline was injected intracerebrally into guinea pigs and hamsters.

Intraperitoneal inoculations of guinea pigs and mice with 1 ml of 24-hour broth culture were without effect. Inoculation of a rabbit intravenously with 0.5 ml of a 24-hour broth culture showed negative results. There was no evidence of infection at the point of inoculation. This organism appears to be of a very low order of pathogenicity.

Discussion. Repeated examination of the purulent material from the brain abscess failed to show acid-fast organisms. Examination of tissues showed no evidence of tuberculosis or actinomycosis. The organism described was isolated in pure culture several times from the abscess of the brain and finally from the spinal fluid. The evidence available indicates that this organism was the causative agent of the abscess and the meningitis.

Although the organism resembles *C. diphtheriæ* in some of its morphological and cultural characteristics, it differs in the following important respects:

(1) No clubbing and little granule formation was seen when the organism was grown on Loeffler's medium. This is the medium of choice for morphological comparison within this genus.

(2) This diphtheroid grows readily on plain nutrient agar, while the true diphtheria bacillus does not.

(3) No pellicle was produced by the diphtheroid.

(4) No zone of hemolysis was shown when grown on human or rabbit blood agar. Some strains of the true diphtheria organism also show no hemolysis.

(5) This organism resembles the mitis type of diphtheria organism most when grown on tellurite, but is a uniform black with no gray portion.

(6) The fermentation reactions were not typical of the diphtheria organism.

(7) This organism was non-pathogenic to laboratory animals.

It is in accord with all the properties which Bergey's manual lists for *Corynebacterium ulcerogenes* with the exception of sucrose fermentation. *C. ulcerogenes* is listed as pro-

ducing acid with sucrose. The organism herein described does not. *C. ulcerogenes* was once isolated from ulcers of the skin in a man. It was non-pathogenic for animals. This is apparently the first time an organism with the characteristics given has been isolated from a brain abscess or a case of meningitis. It is of peculiar interest because of the low order of pathogenicity exhibited in the case described and in the laboratory animals, but yet possesses what was apparently the ability to cause an abscess and meningitis in a child.

Summary. An organism resembling *Corynebacterium ulcerogenes* was isolated repeatedly in pure culture from a brain abscess and spinal fluid of a 7-year-old child. The morphological and biochemical characteristics of the organism were determined, and found to be culturally identical to *C. ulcerogenes*. It was found to be resistant to penicillin, *in vitro*, but inhibited in growth by sulfanilamide, sulfamerazine, sulfasuxidine, and sulfaguanidine. Sulfamerazine was the most effective. The organism was non-pathogenic to guinea pigs, white mice, rabbits, and hamsters, irrespective of the route of inoculation.

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Fumaric Acid Salts as Hydrogogue Cathartics.*

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The alkali metal salts of certain organic acids, notably tartaric and citric acids, have been used for many years as cathartics and for certain other therapeutic purposes. Since the beginning of the present war, tartaric acid has become difficult to obtain and the supply of other commonly used organic acids has been somewhat curtailed. Hence, a search for satisfactory substitutes has been instituted and fumaric acid has been suggested as a likely possibility. It is similar in chemical structure to tartaric acid, is available in large amounts at a moderate cost, and is a "physiological" substance in the sense that it occurs naturally in animal tissues.[1]

The present study was undertaken to determine the laxative effects of fumarates and is a part of a larger study designed to determine the general effects of the administration of fumarates, in varying doses, to different species of animals. The only other extensive published data on the laxative effects of fumarates found in the literature are those of Gold and Zahm[2] who compared the laxative potency of fumarates, sodium tartrate, and magnesium acid citrate in constipated human subjects and showed that the laxative efficacies of the fumarates of sodium, calcium, and magnesium are approximately the same and that these 3 also possess approximately the same laxative potency as sodium tartrate and magnesium acid citrate, gram for gram.

Methods. The subjects chosen for this study were either ambulant or bed patients hospitalized for chronic disease at the Wayne County General Hospital. Many of them had hypertrophic or rheumatoid arthritis, heart disease, Parkinson's Disease, or other afflictions which required institutional care. All the patients volunteered for this study. Each patient had chronic constipation with a long history of dependence on some form of laxative. The patients were considered constipated after 3 or more days without a bowel movement.

The 4 preparations studied, magnesium fumarate, calcium fumarate, sodium fumarate, and Rochelle Salts, were administered by dis-

* Aided by a grant from Chas. Pfizer and Company, Inc., Brooklyn, New York.

1 Annau, E., *et al.*, *Z. f. physiol. Chem.*, 1935, **236**, 1; 1936, **244**, 105.

2 Gold, H., and Zahm, W., *J. Am. Pharm. Assn.*, 1943, **32**, 173.

TABLE I.
Laxative Effect of Rochelle Salts and Fumarates.

	10 g doses 497 doses in 138 patients					15 g doses 105 doses in 32 patients				
		Satisfactory		Unsatisfactory			Satisfactory		Unsatisfactory	
Salts administered	No. of doses	No. of doses	%	No. of doses	%	No. of doses	No. of doses	%	No. of doses	%
Rochelle Salts	174	94	54	80	46	14	10	71	4	29
Sodium Fumarate	107	55	51	52	48	25	14	56	11	44
Magnesium Fumarate	73	36	49	37	51	39	24	61	15	39
Calcium Fumarate	143	82	57	61	43	26	11	42	15	58

solving the salt in a half glass of water using either 10 g or 15 g doses. Each salt was given to relieve 2 successive periods of constipation. Thus, an opportunity was afforded in the same patient to observe the effect of the same salt at least twice. Each patient received in succession all 4 salts or as many as the period of hospitalization and/or the extent of the constipation would permit. The sequence of administration of the 4 salts was not constant. In those patients who received both the 10 g and the 15 g doses the smaller dose preceded the larger.

A record was kept by the nurse of the date, the salt, the dose, the time the medication was given, the time of the bowel movement, and the type of stool (normal, soft, watery). The results were recorded as excellent, good, poor, or failure. A response was considered *excellent* if one or more loose watery stools resulted within 24 hours after the dose. A response was considered *good* if one or more soft stools resulted within 24 hours. The results were recorded *poor* if a hard stool occurred and a *failure* if no bowel movement followed the medication. Other methods, involving both objective and subjective approaches to evaluation of laxation, have been employed in similar investigations; after careful study, it was decided that the present criteria for estimating the efficacy of the cathartic employed, were best suited to the present experimental conditions.

Of the total of 143 patients studied, 111 patients received doses of 10 g each, 5 patients were given 15 g per dose, and 27 patients first were given doses of 10 g and later doses of 15 g each. The number of doses taken by the different patients varied greatly; some received only one dose while others took as many as 19 doses during a period of 6 months of observation. Seventy-one patients received only one drug, 27 patients received 2 drugs, 14 patients received 3 drugs, and 31 patients received 4 drugs. The average number of single doses per patient was 4.2. There were 497 single 10 g doses given and 105 single 15 g doses, thus making a total of 602.

Results. In Table I are summarized the results of the studies of the laxative effect of Rochelle Salts and the 3 fumarates with both the 10 g and the 15 g dose. For this analysis we have considered that a soft or a liquid stool was a "satisfactory" response. If a hard stool occurred or if no bowel movement followed the medication, the response was considered as "unsatisfactory." It will be observed that the average percentage of satisfactory response following the administration of the fumarates is only slightly less than that of Rochelle Salts, a commonly used hydrogogue cathartic. These data indicate that the laxative effects of the fumarates of sodium, magnesium and calcium are approximately the same. The percentage of satisfactory response is only slightly better with the 15 g dose of fumarates as compared with the 10 g dose of fumarates. From Table I, it appears that Rochelle Salts at the 15 g level are far more effective than any of the other salts or than this salt at the 10 g level. However, the total number of doses (15) seems too small to warrant a final conclusion on this point.

The length of time between the taking of the salt and the first bowel movement varied widely between patients and in the same patient with the same amount of the same medication. The average time elapsing be-

tween taking the fumarates and the first bowel movement was about 6 hours.

The 3 salts, magnesium fumarate, calcium fumarate, and sodium fumarate, are free from toxic effects in the doses which were used.

The data obtained in this study are strikingly similar to those reported by Gold and Zahm, and indicate that the fumarates provide a satisfactory substitute for the tartrates as laxative agents.

Summary. The laxative action of sodium, magnesium, and calcium fumarates has been compared with that of sodium potassium tartrate in 143 chronically constipated patients. The fumarates have been found as satisfactory as Rochelle Salts as a laxative agent.

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A Method for Consistent Induction of Chronic Hyperglycemia with Alloxan.

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The capacity of alloxan for inducing in the experimental animal a state resembling diabetes mellitus[1,2,3] has opened a new and promising approach to the study of the disease. One of the major problems involved in the use of alloxan lies in the inconstant and variable responses of experimental animals to its administration.[1,4,5] Large doses of alloxan frequently cause toxic deaths with hepatic or renal lesions, while smaller doses inconstantly produce chronic hyperglycemia. The experience of Lackey *et al.*[6] is a common one: they observed that 60% of rats injected intraabdominally with 200 mg of alloxan per kg of body weight developed moderate to severe diabetes, while 40% either died during the first 72 hours or failed to become diabetic.

In view of the manifest desirability of standardizing the experimental approach to the study of the chronic hyperglycemic state in rats, means were investigated whereby a high percentage of rats treated with alloxan would develop hyperglycemia, with relatively few deaths and with relatively little tendency to become comatose on ordinary laboratory diets. We have found, in substance, that withdrawal of all food from adult animals for periods of 48 to 60 hours will render them almost uniformly susceptible to the subsequent subcutaneous injection of 175 mg of alloxan per kg of body weight. The chronic hyperglycemia so produced leads to few fatalities in the rat and the tendency toward spontaneous recovery of normal blood sugar levels is greatly reduced as compared with animals made diabetic under other conditions.

The animals used were white rats of the Sprague-Dawley strain which were fed on ordinary pellet ration throughout except where otherwise indicated. The dose of alloxan was 175 mg per kg after correction for the molecule of water in alloxan monohydrate. Alloxan samples from two different commercial sources were used, with no discernible differences in their effects. The final solution used contained 19.9 mg of alloxan per ml and was always made just before use; one ml of this solution for each 100 g of body weight was then administered subcutaneously. In most of the experiments the animals used weighed 200-280 g, and dosages were measured to the

1 Dunn, J. S., and McLetchie, N. G. B., *Lancet*, 1943, **2**, 384.

2 Brunschwig, A., Allen, J. G., Goldner, M. G., and Gomori, G., *J. A. M. A.*, 1943, **122**, 966.

3 Bailey, C. C., and Bailey, O. T., *J. A. M. A.*, 1943, **122**, 1185.

4 Gomori, G., and Goldner, M. G., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 287.

5 Bailey, C. C., Bailey, O. T., and Leech, R. S., *N. Eng. J. Med.*, 1944, **230**, 533.

6 Lackey, R. W., Brinde, C. A., Gill, A. J., and Harris, L. C., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 191.

TABLE I.
Effect of Starvation on Susceptibility of Rats to Alloxan.

Starvation period (days)	Total No. rats	Blood sugar (mg%)			
		> 250	180-250	< 180	Deaths
		No. of rats			
3	20	14	1	1	4
2	60	49	6	3	2
1	44	21	6	17	0
1*	16	3	1	12	0
0	40	5	5	28	2

* Fed *ad lib.* 6 hours before injection of alloxan.

nearest 10 g of body weight. When comparisons were made using different methods of treatment, the attempt was made to keep the ages of the rats in comparable groups as nearly alike as possible. Blood sugar levels were routinely determined before injection of alloxan as well as 5-7 days thereafter.[7]

Table I illustrates the effect of starvation and feeding on the susceptibility of rats to alloxan. The animals are grouped according to the severity of the hyperglycemia, using 180 mg % as the upper limit of normality. The incidence of hyperglycemia in the animals which had been starved for 2 days prior to injection of alloxan was 92% (55 of 60 rats) whereas the incidence of hyperglycemia was but 25% (10 of 40 rats) in animals whose food had not been withheld. Furthermore, those animals which did respond to the drug tended to attain higher blood sugar levels if they had previously been starved. It is of interest to note that only one animal in the group of previously starved animals had normal blood sugar values three months after the onset of hyperglycemia, whereas 4 of 6 rats from the group which had not been starved previous to the injection of alloxan had normal blood sugar levels when tested 3 months after they had been shown to be hyperglycemic. The hyperglycemic animals typically lost weight slowly, and showed pronounced polydipsia, polyuria, and polyphagia. Very few deaths occurred during the observation period of 3-4 months subsequent to the onset of hyperglycemia, even though the ordinary pellet diet was used; despite the prolonged hyperglycemia, insulin was not necessary to keep the animals alive.

We have confirmed the observation in the literature that animals which have not responded to one injection are frequently refractory to reinjection of a similar dose of alloxan.[4,8] In order to determine the relation of this refractory state to the state of nutrition of the animal, 26 rats were chosen at random from animals which had previously failed to respond to alloxan. These animals were all in groups which had not been starved prior to the injection, and at least one month had elapsed since the injection of the first dose of alloxan. They were then starved for 60 hours, given the standard dose of alloxan and examined one week later. Twenty-three of the 26 showed marked hyperglycemia, one animal failed to respond and 2 died. The 23 hyperglycemic animals showed no diminution in blood sugar levels 3 months later.

While it is likely that truly refractory animals do exist, it appears that most of the "refractory" rats so far encountered can be made susceptible to alloxan by withholding food for a suitable period of time.

The duration of the starvation period is not a constant factor; in one experience in which 20 larger, older rats (290-340 g) were used, alloxan did not induce hyperglycemia despite previous starvation for 48 hours. After a week's rest, however, the animals were starved for 72 hours, reinjected with alloxan, and all became markedly hyperglycemic.

Since food administered 6 hours before the injection of alloxan conferred a significant degree of protection against the effects of the drug, certain aspects of this phenomenon were investigated further. Groups of rats were

[7] Reinecke, R. M., *J. Biol. Chem.*, 1942, **143**, 351.

[8] Hard, W. L., and Carr, C. J., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 214.

TABLE II.
Effect of Vitamins, Glucose, and Epinephrine on Production of Alloxan Diabetes in Starved Rats.

Treatment	Blood sugar (mg%) > 250	180-250	< 180	Deaths
	No. of rats			
None	7	2	1	0
Vit. (B comp. + C)	7	0	0	3
Glucose*	5	4	1	0
Glucose†	3	0	7	0
Epinephrine	2	2	6	0

* Alloxan injected 1 hr after administration of glucose.
† Alloxan injected 6 hr after administration of glucose.

starved for 48 hours and then given vitamin mixtures, glucose, or epinephrine before injection of alloxan. A typical experiment is summarized in Table II. The rats receiving vitamins each received 200 γ of thiamine chloride, 400 γ riboflavin, 400 γ pyridoxine, 400 γ nicotinic acid, 400 γ *p*-aminobenzoic acid, 400 γ inositol, 1 mg calcium pantothenate and 35 mg choline; other groups of rats received 10 mg ascorbic acid in addition, in a separate injection. The vitamins were administered intraperitoneally 6 hours before the alloxan and, as can be seen, did not protect the animals against the alloxan. Despite the apparent relationship between the B vitamins and other types of experimental diabetes,[9] the vitamins did not protect against the effects of alloxan. This indicates, further, that the increased susceptibility of starved animals to alloxan is not directly related to depletion of vitamin stores.

Glucose was administered as a 25% solution, each rat receiving 4 ml intraperitoneally. Those rats which received alloxan one hour after administration of glucose were not protected against the drug, whereas those which received alloxan 6 hours after injection of the sugar were significantly protected. Blood sugar values of rats in both groups were determined just prior to the injection of alloxan and values of 200-400 mg % were obtained in all cases, indicating that the blood sugar level itself is not the factor determining resistance to alloxan.

The experiments with epinephrine are of particular interest. Each starved rat received 0.1 ml of 1-1000 dilution of epinephrine intraperitoneally, blood samples were immediately drawn, and the alloxan injected. None of the blood sugar levels was higher than 132 mg % yet there was marked protection against the effects of the alloxan. We have repeated this interesting observation many times and have never failed to demonstrate the protective effect of epinephrine despite the fact that the alloxan was administered before any appreciable alteration in blood sugar levels had occurred.

The uniform susceptibility to alloxan evidenced by rats after starvation does not appear to be due to alterations in the liver glycogen, since it has been demonstrated that liver glycogen values in the rat are greater during the second day of starvation than during the first day, probably as a result of increased adrenal cortical activity.[10,11] This increased cortical activity may explain the greater tendency of starved animals to respond to alloxan. Certainly the susceptibility to alloxan bears no simple relationship to blood sugar levels, as is borne out by the experiments in which starved animals were treated with the drug after feeding or after administration of glucose or epinephrine. Detailed histological studies of these effects are in progress.

The action of epinephrine in inhibiting the effect of alloxan was unexpected since the hyperglycemia produced by the epinephrine was never so great as that produced following injection of glucose. Glucose, on the other hand, did not protect against alloxan unless a

9 Gaebler, O. H., and Ciszewski, W. E., *Endocrinol.*, 1945, **36**, 227.

10 Britton, S. W., and Silvette, H., *Am. J. Physiol.*, 1932, **100**, 693.

11 Long, C. N. H., Katzin, B., and Fry, E. G., *Endocrinol.*, 1940, **26**, 309.

period of some hours had elapsed before injection of the drug. While the possibility of direct union between epinephrine and alloxan exists, it hardly seems likely since the drugs were given by different routes of injection, using different syringes. Although a few of the substances which react with alloxan *in vitro* exert some protective effect *in vivo,* the amounts of the protective substances are much greater than the amount of epinephrine which protected against alloxan.[12]

Although Best *et al.*[13] have shown that starvation reduces markedly the insulin content of the pancreas, and this may explain the increased susceptibility of the starved animal to alloxan, the failure of insulin to protect against alloxan when the two are injected simultaneously[14] would indicate the inadequacy of such an explanation.

Summary. 1. Chronic hyperglycemia was produced in 90-95% of adult white rats which had been starved for 48-60 hours and then injected subcutaneously with 175 mg of alloxan per kg. Only 25% of rats which had not been starved became hyperglycemic after injection of a similar dose. The hyperglycemia is persistent and relatively benign; few spontaneous recoveries or fatalities occurred within 3-4 months after injection of alloxan.

2. Fed animals which had not developed hyperglycemia following injection of alloxan became susceptible to subsequent injection of another dose if they were previously starved.

3. Feeding or injecting glucose 6 hours before alloxan diminished the susceptibility of the starved animals to alloxan. Glucose administered one hour before alloxan had no protective effect. Epinephrine, administered immediately before alloxan, protected the starved animals. These effects are not directly related to the blood sugar levels existing at the time alloxan is injected.

4. Mixtures of vitamins of the B complex, and ascorbic acid given 6 hours before injecting alloxan failed to protect starved rats from becoming hyperglycemic.

5. Heavier rats require longer periods of starvation in order to achieve a high degree of susceptibility to alloxan.

[12] Weinglass, A. R., Frame, E. G., and Williams, R. H., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 216.

[13] Best, C. H., Haist, R. E., and Ridout, J. H., *J. Physiol.*, 1939, **97**, 107.

[14] Goldner, M. G., and Gomori, G., *Endocrinol.*, 1944, **35**, 241.

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Preliminary articles are indicated by the letter P after the number of the article. All others are non-preliminary, *i. e.*, complete.